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Loss of tumor suppressor is one of the major mechanisms that lead to tumor formation. We propose to elucidate the mechanism of loss of CEACAM1 tumor suppressor expression in prostate cancer. We found that down-regulation of CEACAM1 expression in prostate tumors is mainly due to transcriptional down-regulation of CEACAM1 gene. We have identified three transcription factors, i.e. AP-2, androgen receptor, and Sp2 that are involved in the regulation of CEACAM1 gene expression. The identification of Sp2 as a transcriptional suppressor of CEACAM1 gene is a novel finding. We found that down-regulation of CEACAM1 gene is mediated by Sp2, which is highly expressed in prostate cancer cells. Inhibitors of histone deacetylase dramatically potentiate CEACAM1 expression in prostate cancer cell lines suggesting that Sp2 inhibits CEACAM1 gene expression through recruitment of histone deacetylase. Thus, loss of CEACAM1 tumor suppressor gene expression in prostate cancer is due to aberrant chromatin acetylation. Results from this study will allow us to better understand the regulation of CEACAM1 gene during tumorigenesis and this may lead to design new therapy strategies to alter tumor progression or to implement early detection and prevention strategies.

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Title: Tumor-Specific Regulation of CEACAM1 Cell Adhesion Molecule in Prostate Carcinogenesis

Principal Investigator: Sue-Hwa Lin, Ph.D.

(4) INTRODUCTION

This is the final report on the project "Tumor-Specific Regulation of C-CAM1 (renamed as CEACAM1) Cell Adhesion Molecule in Prostate Carcinogenesis". We will list the progress for each Specific Aims. As describe below, the most significant contribution of this study is that we have unraveled a new tumor-specific transcriptional regulation mechanism of CEACAM1. The notion that down-regulation of a tumor suppressor gene is due to an increase in the level of a transcription factor that suppresses its expression is novel. In addition, the transcriptional suppressor that we have identified, i.e., Sp2, is also a novel molecule. This is the first time that a specific function for Sp2 is identified. A manuscript on this discovery has been written and is being submitted for publication. We have attached the manuscript as part of our progress report.

CEACAM1 is a 105 kDa glycoprotein which mediates homotypic cell adhesion. We have shown that CEACAM1 plays critical roles in prostate cancer initiation and progression and that loss of CEACAM1 is an early event in the development of prostate cancer. We showed that re-introduction of CEACAM1 into prostate cancer cells could reverse their cancerous growth. In addition, we also showed that reduction of CEACAM1 expression, by CEACAM1 antisense transfection, in non-tumorigenic prostate epithelial cells (NbE) induced their tumorigenicity. These results indicate that CEACAM1 is a tumor suppressor and CEACAM1 plays a critical role in pathways that regulate the progression of prostate cancer. Thus, CEACAM1 itself or drugs that up-regulate CEACAM1 expression could be promising therapeutics for prostate cancer treatment.

The mechanism of CEACAM1 down-regulation in tumorigenesis is not clear. There is no evidence that the human CEACAM1 gene, which is located on chromosome 19, is deleted in prostate cancer patients. Decreased expression but not mutation of CEACAM1 is detected at the early stages of prostate carcinogenesis. Therefore, we believe that down-regulation instead of irreversible loss of CEACAM1 expression in vivo is the major cause of tumorigenesis. We propose to **identify mechanisms that regulate CEACAM1 gene expression in prostate carcinogenesis.** Specifically, we plan to (1) examine whether altered chromatin remodeling

contributes to CEACAM1 down regulation; (2) study interactions between known transcription factors and CEACAM1 promoter; specially examine the involvement of AP-2 in CEACAM1 gene expression in tumorigenesis; (3) screen for factors that modulate CEACAM1 expression. The proposed work was divided into three Tasks to be carried out in parallel.

Task 1. Determine whether altered chromatin remodeling contributes to CEACAM1 down regulation (months 1-12)

Task 2. Study interaction between known transcription factors and CEACAM1 promoter, specifically AP-2. (Months 1-18)

Task 3. Search for the transcriptional activators/co-activators involved in CEACAM1 expression (months 1-24)

Task 4. Search for transcriptional repressors that regulate CEACAM1 expression (Months 1-24)

Results from this study will allow us to understand CEACAM1 gene regulation during tumorigenesis. This study may also yield targets for the design of new strategies to control tumor progression or to implement early detection and prevention. While gene therapy using CEACAM1 is a promising approach, drugs that can activate CEACAM1 gene expression is more convenient and can be used in conjunction with CEACAM1 gene therapy to achieve a better therapeutic efficiency. Therefore, our research will contribute to the improvement of various aspects of prostate cancer control.

(5) BODY

5.1. Studies performed under Task 1— Determine whether altered chromatin remodeling contributes to CEACAM1 down regulation

5.1.1. Rationale: The conformation of genes within chromatin determines whether a gene is in its active or inactive state. These structural features are regulated by enzymes that modify chromatin structure. Histone acetylation leads to open chromatin conformation that promotes gene transcription by making promoter sequences accessible to transcription factors. We plan to investigate, using the chromatin immunoprecipitation (ChIP) assay, whether changes in chromatin remodeling might account for CEACAM1 down regulation in tumorigenesis.

5.1.2. Experimental Plan: The ChIP assay will be used to assess whether chromatin remodeling is involved in CEACAM1 promoter inactivation in tumorigenesis. Ac-histone in chromatin can be cross-linked to the associated DNA in vivo. Following immunoprecipitation with anti-acetylated Histone 4 (AcH4) antibody, the associated DNA can be analyzed by polymerase chain reaction (PCR). NbE cells (CEACAM1 expressing normal prostate epithelial cells) and Mat-LyLu (MLL) cells (a metastatic prostate cancer cell line expressing only 4% of CEACAM1 compared with NbE cells) will be analyzed for the extent of the acetylated H4 association with their CEACAM1 promoter. The advantage of the ChIP assay is that chromatin conformation of native promoters can be analyzed. This assay will allow us to address whether histone deacetylases might be involved in transcriptional down-regulation of the CEACAM1 gene in prostate cancer cells and which region of the CEACAM1 promoter might be involved.

5.1.3. Results: Quantitative ChIP assay were carried out as described (1,2) using the ChIP assay kit (UBI). NbE or Mat-LyLu cells grown on 10 cm culture dishes were treated with formaldehyde to a final concentration of 1% at 37°C for 10 min. The cells were scrape from the plates in phosphate-buffered saline (PBS) containing protease inhibitors. The cell pellets were resuspended in lysis buffer and sonicated to reduce DNA fragments to 200 to 1000 bp. The cell debris were removed by centrifugation and the supernatant that contained the chromatin were saved for further analysis. Aliquots of these chromatin preparations were used to determine the total DNA content, which was then used to normalize the PCR signals. The remaining chromatin solution was used for immunoprecipitation. Anti-acetyl histone H4 antibody were added to chromatin solution and incubate overnight. The immune complexes were collected with protein-A agarose. The protein-A agarose beads were pellet by centrifugation and the immune complexes eluted by elution buffer. The crosslinked protein-DNA complexes were incubated at 65°C for 4 hours to reverse the crosslink. The proteins were digested with proteinase K and DNA recovered by phenol/chloroform extraction and ethanol precipitation. The genomic DNA fragments bound to anti-acetylated histone antibody were analyzed by quantitative PCR using primer pairs corresponding to different regions of the CEACAM1 promoter. Significant increase (about 2-fold) in the amount of chromatin from the CEACAM1 promoters associated with anti-acetylated histone antibody was observed in the NbE cells compared to that of Mat-LyLu cells (Fig. 1). These results suggest that CEACAM1 promoter region is in more "acetylated" form and thus more "active" state in NbE cells compared to that of Mat-LyLu cells.

5.1.4. Discussion Preliminary results support the notion that chromatin remodeling is involved in down-regulation of CEACAM1 in tumorigenesis. This observation will be further

confirmed by using real time PCR to quantitate the difference.

5.2. Studies performed under Task 2— Study interaction between known transcription factors and CEACAM promoter, specifically AP-2.

5.2.1. Rationale: The CEACAM1 promoter lacks a TATA or CAAT box but has potential binding sites for known basal and regulatory transcription factors. Several AP-2 (activator protein 2) binding sites were found in the CEACAM1 promoter. AP-2 (activator protein 2) was shown to regulate the expression of several oncogenes and tumor suppressor genes and has been shown to involve in tumorigenesis. Thus, AP-2 is one of the known factors that may be potentially involved in CEACAM1 down-regulation in tumorigenesis.

5.2.2. Experimental Plan: We examined whether AP-2 has any effect on CEACAM1 promoter activity by co-transfecting CEACAM1 promoter-luciferase constructs and an AP-2 expression vector into MLL cells.

5.2.3. Results: Addition of AP-2 resulted in an 8-10-fold increase in luciferase expression when the reporter gene was driven by the -1609, -459, -249, -194 bp of CEACAM1 promoter (Fig. 2). Deletion of the CEACAM1 promoter down to -147 bp abolished the AP-2 effect. This result suggests that AP-2 is a transcriptional activator of CEACAM1 and the AP-2 responsive element is located between -194 bp and -147 bp region of the CEACAM1 promoter, which is consistent with prediction from promoter sequence analysis.

5.2.4. Discussion: These results suggest that AP-2 is a transcriptional activator of CEACAM1 and thus is a potential regulator of CEACAM1 expression during tumorigenesis. To further test this notion, we will study whether AP-2 expression is altered in prostate cancer cells by Northern blot analysis. If AP-2 shows a similar pattern of down-regulation as that of CEACAM1, it is very likely that it plays a role in the reduced CEACAM1 expression during carcinogenesis. To assess the ability of AP-2 to activate endogenous CEACAM1 gene expression, AP-2 will be transfected into prostate cancer cells. The functional consequence of AP-2 expression will be examined by testing whether these AP-2 expressing prostate cancer cells have lower *in vivo* tumorigenicity in nude mice.

5.3. Studies performed under Task 3— Search for the transcriptional activators/co-activators involved in CEACAM expression

5.3.1 Rationale: We hypothesized that decreased CEACAM1 transcription during tumorigenesis may be due to a loss of activator(s) or coactivator(s). Such a factor should have the following properties: It should (1) exhibit different levels of expression between normal and cancer cells,

(2) be able to activate CEACAM1 expression, and (3) suppress tumorigenicity of prostate cancer cells. We will identify the potential tumor-specific transcription factors based on these functional criteria.

5.3.2. Experimental plan: We plan to functionally identify these factors in the context of the native CEACAM1 promoter, using a tumor cell line with a low level of CEACAM1 expression such that modulation of CEACAM1 expression can be easily detected. Since CEACAM1 is a membrane protein, cells that have increased CEACAM1 expression can be selected by CEACAM1 antibody binding followed with fluorescence activated cell sorting (FACS). The Dunning rat prostate cancer cell lines have the properties suitable for our purpose. Dunning 3327 prostate cell line was isolated from rat prostate tumor by Dunning (3) from inbred Copenhagen male rat. Sublines with different biological characteristics are available (4), which represent tumors ranging from relatively benign, slow growing, differentiated, and androgen-sensitive tumors to rapidly growing, anaplastic (AT-2), and hormone-insensitive malignant tumors (AT-3.1 and Mat-LyLu). We first characterized CEACAM1 expression in these cell lines to determine if CEACAM1 protein expression levels show distinct tumor-specific down-regulation.

5.3.3. Results

5.3.3.1 Western immunoblot analysis of CEACAM1 protein expression in the Dunning series prostate cancer cell lines: As shown in Fig. 3, distinct changes in CEACAM1 protein expression in the Dunning cell lines are observed by western blot analysis. A significant decrease in CEACAM1 protein levels occurred at the transition from normal cells (NbE) to carcinoma, AT-2 and AT-3.1, followed with further reduction in rapidly growing tumors, Mat-LyLu (MLL). A normal prostate cell line NbE, derived from ventral prostate of Noble rat (5), was used as a control. In Mat-LyLu cell line, CEACAM1 expression level is about 4% as compared to that of the NbE control cell line. MLL cell line was selected for further studies.

5.3.3.2 Transfection of human prostate cDNA library in mammalian expression vector into Mat-LyLu and FACS analysis: To screen for CEACAM1 activation factors, a human prostate cDNA library in a mammalian expression vector was constructed and used to transfect Mat-LyLu cells. Three million Mat-LyLu cells were transfected with 15 ug of expression vector using lipofectamine (Gibco/BRL). At 2 days post-transfection, these cells were trypsinized from the plates and incubated with polyclonal anti-CEACAM1 antibodies followed with FITC-conjugated secondary antibody. The top 2% fluorescence positive cells, which were considered CEACAM positive, were separated from total cell populations by FACS.

5.3.3.3 Isolation of plasmid DNA from CEACAM positive cells: Total DNA was isolated from the FACS sorted cells and electroporated into E. coli, which were selected in ampicillin-containing agar plates (library plasmids contain β-lactamase). Ampicillin-resistance colonies were recovered and pooled, and their plasmid DNA (first round DNA) (15 ug) were used to transfect seven million MLL cells. The top 4.5% CEACAM1 positive cells were isolated by FACS, and these plasmid DNAs (second round DNA) were retrieved as above. This selection procedure was repeated two more times and the resulting DNAs were analyzed by restriction enzyme digestion.

5.3.3.4 Results from 4 cycles of FACS selection: As shown in Fig. 4, the original prostate cDNA library contained DNA inserts with various sizes and appeared as a smear (lane 2). Enrichment of certain insert sizes was apparent following four-cycle selection (lane 6). Plasmids from the fourth selection were transformed into bacteria and plasmids from single bacteria colonies were isolated and their sequences will be determined. These plasmids will be transfected into MLL to confirm their ability to activate CEACAM1 gene promoter. Plasmids that consistently activate CEACAM1 expression in MLL cells will be further analyzed.

5.3.4 Discussion: The concept of functional screening of molecules that modulate CEACAM1 expression in tumor cells was considered to be novel, but risky, by the reviewers. With the funding, we were able to evaluate the method and improve the efficiency and accuracy of the steps taken. Our modifications include (1) Use of the rat MLL cells instead of human prostate cancer cell lines. The reason is that the CEACAM1 antibodies react with rat CEACAM well but only react weakly with human CEACAM1. This cell line also allows us to study CEACAM1 gene regulation in its natural context. (2) We have compared several transfection methods/reagents and found that lipofectamine can produce about 30% transfection efficiency in MLL cells. (3) Electroporation of isolated plasmids into competent E. coli greatly increases the efficiency of transformation. This step not only amplifies selected plasmids, but also removes mitochondria or cellular DNA that are present in the DNA preparation. With these improvements, we are confident that factors that modulate CEACAM1 expression can be isolated.

5.4. Studies performed under Task 4 — Search for transcriptional repressors

5.4.1. Rationale: Down-regulation of CEACAM1 expression during prostate cancer progression may also arise from increase of factors that suppress CEACAM1 expression.

5.1.3. Experimental Plan: CEACAM1 promoter regions that show different activity when expressed in normal versus tumor cell lines will be used to probe for the transcription factor that bind to that region differentially.

5.4.3. Results

5.4.3.1. CEACAM1 Messages in Prostate Cancer Cell Lines

As shown in Fig. 3, CEACAM protein was down-regulated in Mat-LyLu cell line. Since decrease in protein expression can arise from transcriptional and post-transcriptional events, we further examine the levels of CEACAM1 messages in the NbE and Mat-LyLu cell lines. Using a probe generated from full-length CEACAM1 cDNA (6), Northern blotting showed a significant decrease in the steady-state levels of 4 kilobase (kb) mRNA for CEACAM1 in Mat-LyLu cells (Fig. 5). These observations indicate that loss of CEACAM1 protein is due to the reduction of CEACAM1 transcript. Thus, down-regulation of CEACAM1 expression in prostatic cancer cells could be due to altered transcriptional activity, which could result from changes in the *cis* elements, e.g., methylation, in the CEACAM1 promoter or the *trans* factors, e.g., transcription factors, that regulate promoter activity.

5.4.3.2. Promoter Methylation

Aberrant hypermethylation of 5' CpG islands within proximal promoter regions has been implicated as a mechanism by which tumor suppressor genes can be inactivated. Examples of this mechanism have been best demonstrated for the VHL and p16 tumor suppressor genes (7,8). In addition, Graff et al. (9) have also shown that hypermethylation is one of the mechanisms for the down-regulation of the "metastasis suppressor" gene, i.e., E-cadherin, in prostate cancer. Genomic clone containing the CEACAM1 promoter has been isolated (10). Promoter sequence analysis showed that CEACAM1 promoter does not have high CpG content typical for genes whose expressions were modulated by methylation. Only 5 CpG dinucleotides were found within the 250 bp promoter proximal region of CEACAM1 promoter. Treatment of the Mat-LyLu cells with DNA methylation inhibitor 5-aza-2'-deoxycytidine did not result in an increase of CEACAM1 protein expression as judged by RT-PCR and Western blot analysis (data not shown). Thus, promoter methylation was unlikely to be involved in CEACAM1 regulation. This observation is also consistent with the report by Rosenberg et al. (11) that methylation was not detected in mouse CEACAM1 gene in colon carcinoma samples examined. Instead, hypomethylation of mouse CEACAM1 gene was detected in one of three colon carcinoma samples examined.

5.4.3.4. Transcriptional regulation of CEACAM1 gene expression in prostatic cancer cell lines

Since CEACAM1 down regulation seems to occur at the transcriptional level, we next

examine how transcriptional regulation of CEACAM1 gene occurs in tumorigenesis. We have previously isolated a genomic CEACAM1 gene (10). The promoter region of this gene (1.6 kb) was cloned in front of a luciferase reporter gene and a series of transfection studies were performed to localize the promoter sequence. Using promoter deletion analysis, the minimal promoter is located between -194 and -147 bp 5' of the CEACAM1 translation start site (10).

In order to compare promoter activities in the CEACAM1 positive and negative cell lines, we transfected the CEACAM1 promoter-luciferase constructs into NbE and Mat-LyLu cells. Since the tumor-specific regulatory elements may reside outside the minimal promoter, we tested the activity of -1609 bp, -439 bp, -249 bp and -194 bp promoter constructs. The transfection efficiencies were normalized against the Renilla luciferase activity from pTK-Ren, in which the Renilla luciferase expression was driven by a thymidine kinase promoter. As shown in Fig. 6, a similar pattern of promoter activity was observed in the four promoter constructs tested in the Mat-LyLu cells as compared with the NbE cells. However, there is a significant decrease, about 4-fold, in the overall CEACAM1 promoter activity in Mat-LyLu cells. These results suggest that down-regulation of CEACAM1 in prostatic cancer cells may be due to altered transcription factors that regulate CEACAM1 promoter activity and that one of the tumor-specific promoter activities lies between -194 and -147 bp in the minimal CEACAM1 promoter region.

5.4.3.5. Binding of NbE and Mat-LyLu nuclear extract to CEACAM1 promoter

Because loss of transcriptional activator(s) or activation of transcriptional repressor(s) could be one of the mechanisms responsible for CEACAM1 down-regulation in tumorigenesis, gel shift assays were used to examine the binding of nuclear proteins from NbE or Mat-LyLu cells to an oligonucleotide containing the minimum promoter sequence (nt -194 to -147, 194-probe) of the CEACAM1 promoter. As shown in Fig. 7, three major bands (complex II to IV) were detected in the NbE nuclear extract. The binding of complex II-IV is specific as they can be competed by excess of unlabeled 194probe. In contrast, four major bands (complex I-IV), which can be specifically competed by excess of unlabeled 194probe, were detected in the Mat-LyLu nuclear extract (Fig. 7). Because complex I is only present in the Mat-LyLu extract, we investigated the possibility that the protein(s) present in complex I may be related to the decreased CEACAM1 promoter activity in Mat-LyLu cells.

5.4.3.6. Involvement of Sp transcription factor family at the CEACAM1 promoter

Sequence analysis of 194-probe indicates that this region of CEACAM1 promoter is highly GC-rich and it contains elements that match the consensus sequence for Sp1 binding site (10). Thus, it is likely that these complexes contain Sp family of transcription factors. EMSA supershift using antibody against the Sp1, Sp2, Sp3, and Sp4 was used to identify the protein(s)

present in complex I-IV in Mat-LyLu cells. As shown in Fig. 8, complex I, which is present in nuclear extracts from Mat-LyLu but not NbE cells, was shifted by anti-Sp2 antibody as judged by the decrease of complex I intensity (lane 5). Complex II was shifted by anti-Sp3 antibody. Anti-Sp1 antibody generated a weak supershifted band, however, no significant decrease was observed in any of the complexes, suggesting Sp1 might be a minor component in these complexes. It is very likely Sp1 is associated with complex II as it was reported that Sp1 and Sp3 competed for the same binding sites in vivo (12). Since Sp2 appears to be the major protein in complex I, it is a candidate transcription factor involved in the decreased CEACAM1 promoter activity in Mat-LyLu cells.

5.4.3.7. Nuclear protein levels of Sp2 correlate with CEACAM1 down-regulation

Down-regulation of CEACAM1 in Mat-LyLu cells might be due to an increase in the nuclear protein concentrations of Sp2 in Mat-LyLu (CEACAM1 low-expressing) as compared with NbE (CEACAM-1 high-expressing) cells. Western blot analysis was performed on cell extracts derived from Mat-LyLu or NbE cells using antibodies against Sp2. Protein levels of Sp2 was about 10-fold higher in Mat-LyLu cells than NbE cells (Fig. 9).

Immuno-localization of Sp2 showed that they are mainly localized in the nucleus of both Mat-LyLu and NbE cells (Fig. 10). Consistent with western blot analysis, the amount of Sp2 protein is higher in the nucleus of Mat-LyLu cells compared with that of NbE cells by both immunofluorescence and immunoperoxidase detection using DAB (Fig. 10). Thus, the relative levels of Sp2 in Mat-LyLu versus NbE cells correlate with potential repressive functions of Sp2 in down-regulating the CEACAM1 promoter in these cells.

5.4.3.8. Sp2 binding sequence at the CEACAM1 promoter

Sp-family transcription factors were known to bind to G-rich elements such as the GC-box and the related GT/CACCC-box (12). Sp2 was cloned because it binds in vitro to a GT-box promoter element within the T-cell antigen receptor □ gene (13). The 194 probe contains a GT-box like sequence between nt-173 to -164 and thus, this GT-box like sequence may be the potential Sp2 binding sequence. These nucleotides were mutated to generate a 194-mut probe and used as a competitor in EMSA. Addition of 100-fold excess of the 194-mut probe blocked complex II-IV formation, but did not alter complex I formation (Fig. 8, lane 8). Because the 194-mut probe cannot compete for Sp2 binding to complex I, this observation suggests that Sp2 may bind to the GT-rich sequence between nt-173 to -164 in the CEACAM1 promoter.

5.4.3.9. Mutation of Sp2 binding site increases promoter activity

Because mutation of nt -174 to -164 of CEACAM1 promoter resulted in a loss of Sp2 binding, a -194pLuc reporter containing the Sp2 site mutation (-194mutpLuc) was used to

determine if loss of Sp2 binding results in an increase in CEACAM1 promoter activity. The -194mutpLuc reporter construct was transfected into Mat-LyLu cells and its promoter activity compared with that of wild type -194pLuc reporter construct. Mutation of the Sp2 binding site reproducibly resulted in a 7 to 14-fold increase in CEACAM1 promoter activity in Mat-LyLu cells compared to that of wild-type promoter (Fig. 11), suggesting that binding of Sp2 suppresses CEACAM1 promoter activity in prostate cancer cells.

5.4.3.10. Inhibition of HDAC activity by TSA activates CEACAM1 promoter activity

Association with histone deacetylase (HDAC) contributes to the suppressive activity of several transcription factors. To investigate whether the inhibitory effect of Sp2 on CEACAM1 promoter activity in Mat-LyLu cells was mediated by HDAC, the HDAC inhibitor trichostatin A (TSA) was used to examine if it can relieve Sp2-mediated repression at the CEACAM1 promoter. Mat-LyLu cells were transfected with -194pLuc or -194mutpLuc and treated with or without TSA. Treatment of cells with TSA resulted in about 19-fold increase of -194pLuc activity while TSA did not have an effect on -194mutpLuc activity (Fig. 12). These results indicating that Sp2 recruits HDAC to repress CEACAM1 transcription.

5.4.4. Discussion

We demonstrate that suppression of CEACAM1 transcription activity by Sp2 is one of the mechanisms that down-regulate CEACAM1 expression in prostate cancer and Sp2 represses CEACAM1 transcription through recruitment of HDAC. Decreased expression of tumor suppressors is commonly observed in tumors. Although promoter silencing by methylation has been demonstrated to be one of the mechanisms, only few tumor suppressors were shown to be regulated by promoter methylation. Our study provides evidence that increased expression of transcription suppressors in tumor cells constitute another mechanism for the regulation of tumor suppressor expression in tumorigenesis. Although this mechanism is only been demonstrated with CEACAM1, it is likely to be applicable to other tumor suppressors.

(6) KEY RESEARCH ACCOMPLISHMENT

- Chromatin remodeling is involved in the regulation of CEACAM1 expression in prostate tumorigenesis
- AP-2 is one of the transcription activator for CEACAM1 gene expression
- The transcription factor Sp2 plays a role in the down-regulation of CEACAM1 gene in prostate tumorigenesis
- Sp2 recruits histone deacetylase to down-regulate CEACAM1 gene expression

(7) REPORTABLE OUTCOMES

1 manuscript published: Phan, D., Sui, X., Luo, W., Najjar, S., Jenster, G., and **Lin, S.-H.**: Androgen regulation of the Cell-Cell Adhesion Molecule-1 (CEACAM1) gene. Mol. Cell. Endo., **184,** 115-123 (2001).

1 manuscript to be submitted: Phan, D., Tunstead, J., Galfione, M., Luthra, R., Najjar, S. M., Yu-Lee, L.-Y., and Lin, S.-H.: Tumor Specific Regulation of CEACAM1 Gene Expression by Sp2 and Histone Deacetylase. submitted.

1 abstract presented at 10th International Workshop of CEA family genes (Sept. 2-5, 1999): Phan, D., Jenster, G., Luo, W., Sui, X., Najjar, S., and Lin, S.-H.: Transcriptional regulation of CEACAM1 gene by androgen receptor.

(8) CONCLUSIONS:

We propose to elucidate the mechanisms that regulate CEACAM1 gene expression in prostate carcinogenesis. We have identified at least two transcription factors, i.e. AP-2 and androgen receptor, that are involved in the up-regulation of CEACAM1 gene expression and one transcription repressor, i.e. Sp2, that specifically down-regulates CEACAM1 promoter activity in tumor cells. In addition, we have developed a novel *in vivo* functional screening method to identify new transcription factors that regulate CEACAM1 gene expression during prostate carcinogenesis. Results from this study will allow us to better understand the regulation of CEACAM1 gene during tumorigenesis and this may lead to design new therapy strategies to alter tumor progression or to implement early detection and prevention strategies.

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Dillon Phan
Jim Tunstead

(11) APPENDICES:

Figures 1 – 12

1 reprint: Phan, D., Sui, X., Luo, W., Najjar, S., Jenster, G., and **Lin, S.-H.**: Androgen regulation of the Cell-Cell Adhesion Molecule-1 (CEACAM1) gene. Mol. Cell. Endo., **184,** 115-123 (2001).

1 manuscript to be submitted: Phan, D., Tunstead, J., Najjar, S. M., Yu-Lee, L.-Y., and Lin, S.-H.: Tumor Specific Regulation of CEACAM1 Gene Expression by Sp2 and Histone Deacetylase.

Fig. 1. Chromatin remodeling at the CEACAM1 Promoter. (A) Our hypothesis is that more acetylated (Ac) histones are associated with the CEACAM1 promoter in normal prostate epithelial cells, and deacetylation of the histones associated with CEACAM1 promoter may occur during prostate tumorigenesis and give rise to transcriptional down regulation of the CEACAM1 gene. The chromatin immunoprecipitation (ChIP) assay will be used to test this hypothesis. Acetylated (Ac) histone in chromatin can be cross-linked to the associated DNA in vivo. The DNAs are sonicated to reduce fragments to 200 to 1000 bp. Following immunoprecipitation of the DNA/histone fragments with anti-acetylated Histone 4 Ab (Y), the associated DNA can be analyzed by PCR.

(B) PCR of DNA fragments immunoprecipitated by anti-acetylated Histone 4 antibodies.

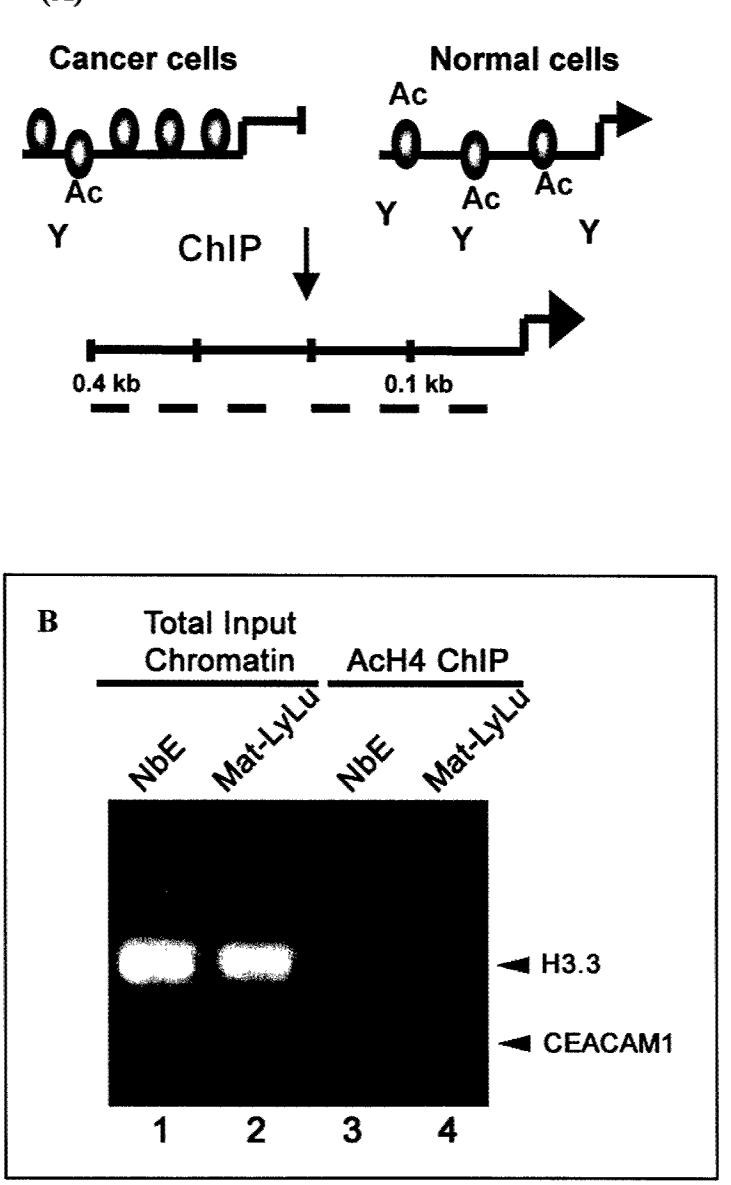


Fig. 2. Regulation of the CEACAM1 expression by AP-2. A series of reporter plasmids containing CEACAM1 promoter fragments with different 5' deletions were co-transfected with mammalian expression vector containing the AP-2 gene into Mat-LyLu cells. Luciferase activities of these cell lysates were determined and reported as averages ± S. D. in relative light units from triplicate transfections.

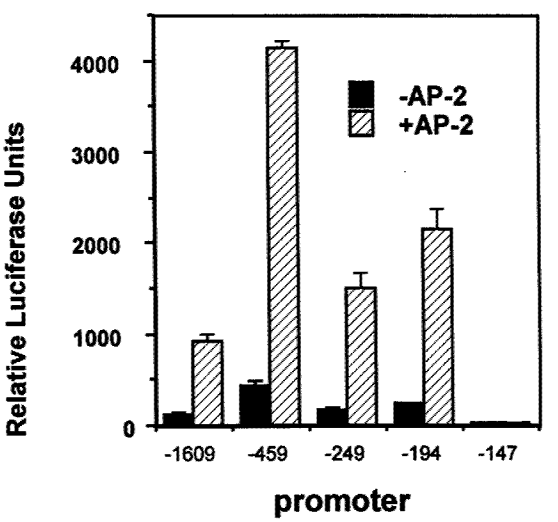


Fig. 3. Western immunoblot analysis of CEACAM1 expression in Dunning rat prostate cancer cell lines. The levels of CEACAM1 protein expression in Dunning rat prostate cancer cell lines (AT2, AT3.1, and Mat-Ly-Lu (MLL)) were examined by western immunoblot analysis using antibodies against CEACAM1. A normal prostate cell line NbE, derived from ventral prostate of Noble rat, was used as a control. A significant decrease in CEACAM1 protein levels occurred at the transition from normal to carcinoma (i.e., AT2, and AT3.1), followed with further reduction in CEACAM1 protein level in the metastatic subline MLL. In the MLL cell line, the CEACAM1 expression level is about 4%, compared with that of normal control cells.

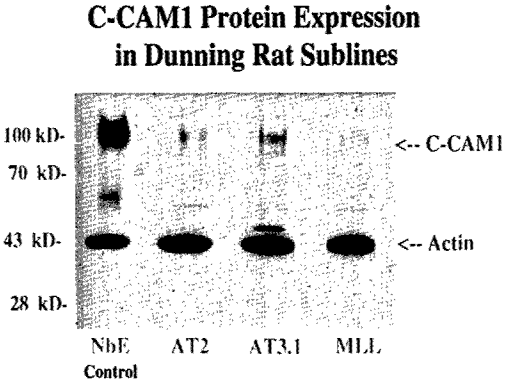


Fig. 4. Restriction digest profile of the plasmids isolated from cells selected from FACS. The original prostate cDNA library contained DNA inserts of various sizes and appeared as a smear (total DNA). Enrichment of certain insert sizes was apparent following four-cycle selection.

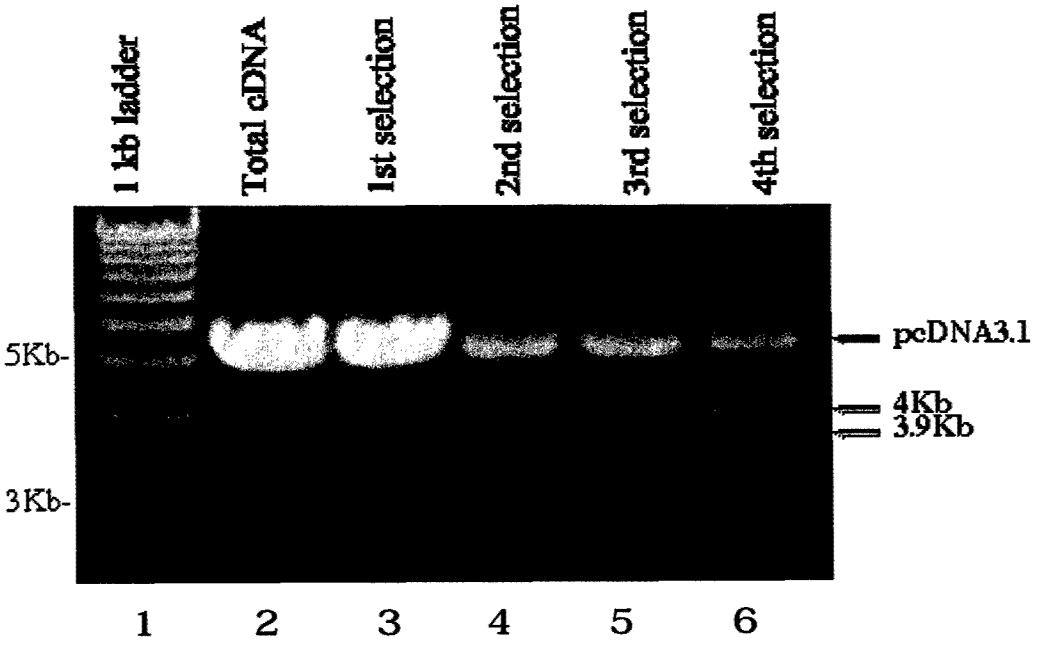


Fig. 5. Absence of CEACAM1 messages in prostate cancer cell lines. To distinguish whether decrease in CEACAM1 protein expression is due to transcriptional or post-transcriptional events, the levels of CEACAM1 messages in the NbE, AT2, AT3.1, and Mat-LyLu cell lines were tested by RNase protection using a probe generated from the full-length CEACAM1. Northern blot analyses showed that the three prostate cancer cell lines contain only detectable amounts of CEACAM1 message while RNA from NbE cells, which was previously shown to express CEACAM1, showed strong signal of CEACAM1 message.

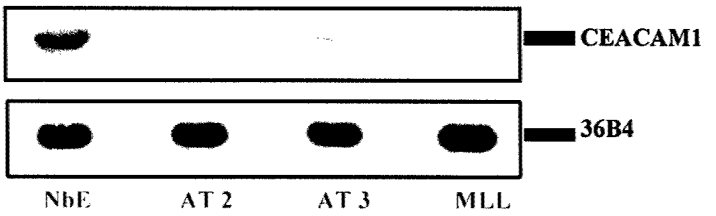


Fig. 6. Decreased CEACAM1 promoter activity in low CEACAM1 expressing cells. The CEACAM1 promoter-luciferase constructs were transfected into NbE (CEACAM1 positive) and Mat-LyLu (CEACAM1 negative) cells. We tested the activity of a series ofvarious length of CEACAM1 promoters. CEACAM1 promoter (-1609 bp) cloned in reverse orientation, -1609RevpLuc, was used as a reference. The transfection efficiencies were normalized against the luciferase activity from pTK-Renilla, in which Renilla expression was driven by a thymidine kinase promoter. Decreases in transcriptional activity were observed in Mat-LyLu prostatic cancer cell line as compared to the NbE cells.

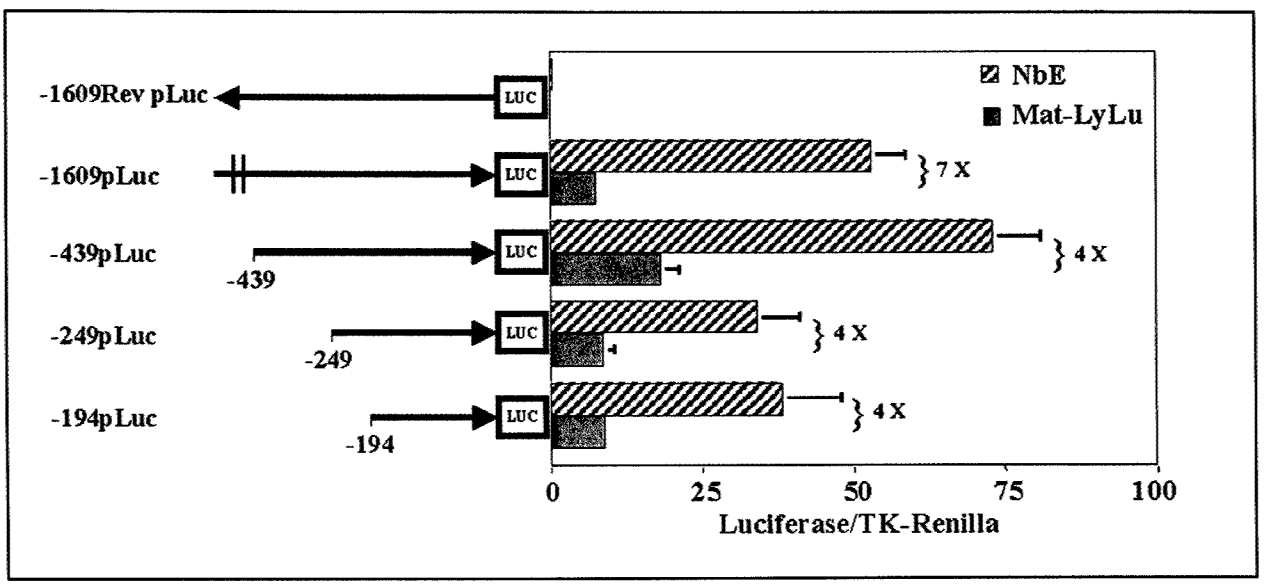


Fig. 7. Electromobility Shift Assay. Oligonucleotides having sequences corresponding to the region between -194 to -147 bp of the CEACAM1 promoter were synthesized and used as probe (194 probe). Nuclear extracts from the NbE or Mat-LyLu were used. Four protein-DNA complexes are indicated by arrows.

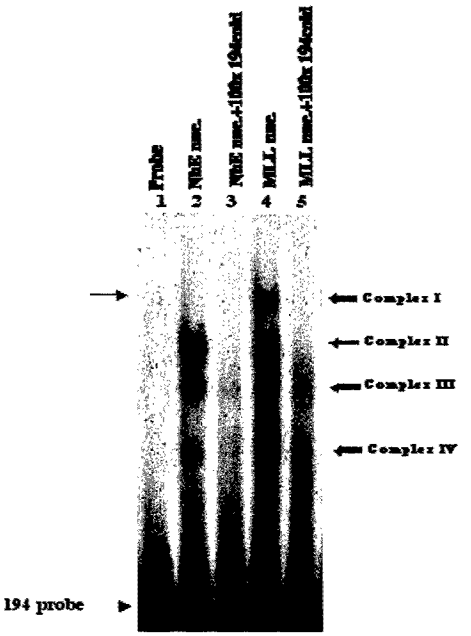
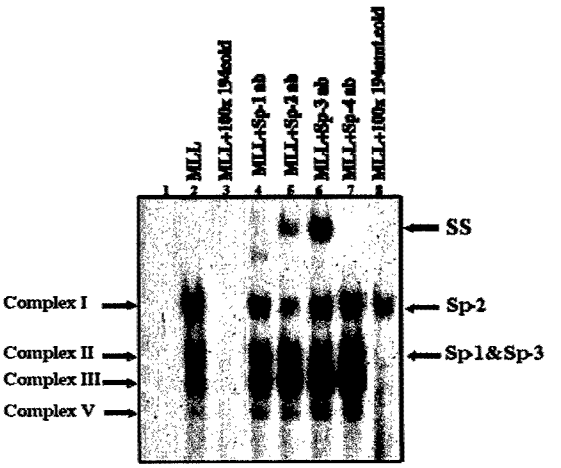


Fig. 8. Evidence that complex I contains transcription factor Sp2. Gel shift analysis using 194 probe was performed in the absence or presence of antibodies against Sp1, Sp2, Sp3 or Sp4. Complex I was shifted by anti-Sp2 antibody as judged by the decrease of complex I intensity (lane 5). Complex II was shifted by anti-Sp3 antibody. Anti-Sp1 antibody generated a weak supershifted band, however, no significant decrease was observed in any of the complexes, suggesting Sp1 might be a minor component in these complexes.



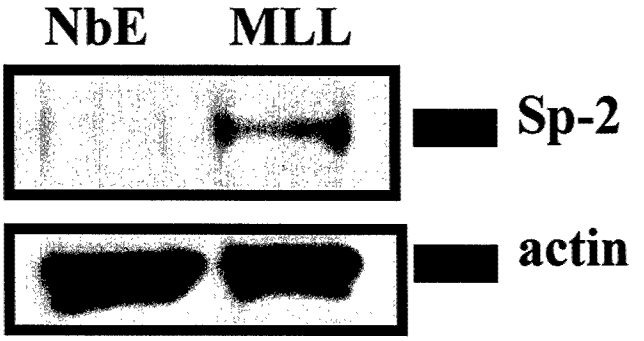


Fig. 9. Differential expression of Sp2 in normal versus cancer cells. Cell lysates (20 ug) prepared from NbE or Mat-LyLu cells were boiled in SDS sample buffer and the proteins were separated by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane and blotted with anti-Sp2 antibodies.

Fig. 10. Nuclear localization of Sp2. Mat-LyLu or NbE cells grown on coverslips were fixed by formaldehyde and immunostained with antibody against Sp2. The Sp2 protein was detected by diaminobezidine.

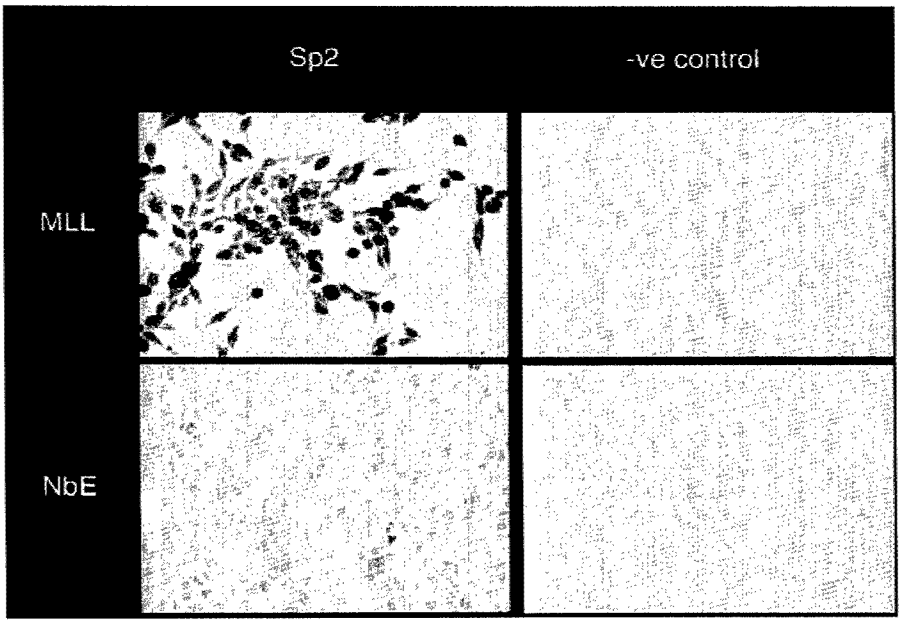


Fig. 11. Mutation of Sp2 binding site increases CEACAM1 promoter activity. Mat-LyLu cells were transfected with luciferase reporter plasmids containing wild-type -194 CEACAM1 promoter (p-194Luc) or -194 promoter with mutation of Sp2 binding site (p-194mutLuc). Fold induction of luciferase activity was calculated relative to that of p-194Luc.

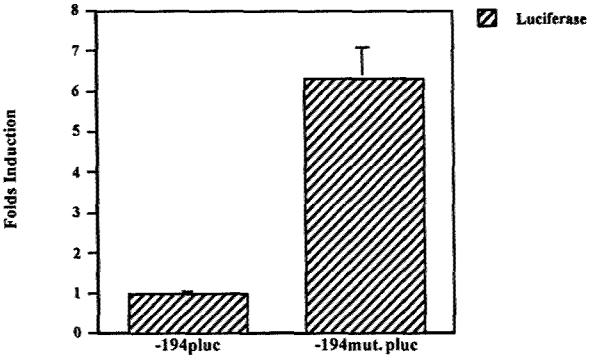
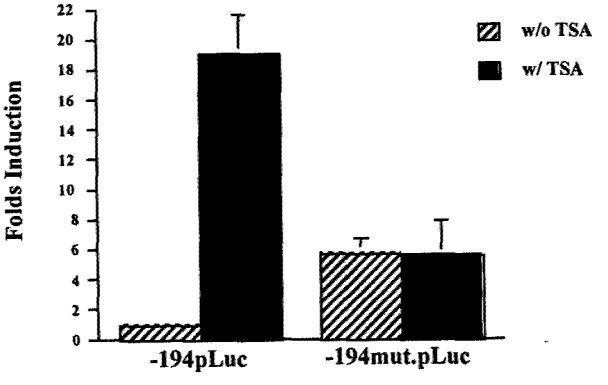


Fig. 12. Effect of trichostatin on CEACAM1 promoter activity. Mat-LyLu cells were transfected with p-194Luc or p-194mutLuc in the presence or absence of 1 uM trichostatin (TSA). Fold induction of luciferase activity was calculated relative to that of p-194Luc in the absence of TSA.



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Androgen regulation of the cell–cell adhesion molecule-1 (*Ceacam1*) gene

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Abstract

Previous studies have established that the cell–cell adhesion molecule-1 (CEACAM1, previously known as C-CAM1) functions as a tumor suppressor in prostate cancer and is involved in the regulation of prostate growth and differentiation. However, the molecular mechanism that modulates CEACAM1 expression in the prostate is not well defined. Since the growth of prostate epithelial cells is androgen-regulated, we investigated the effects of androgen and the androgen receptor (AR) on CEACAM1 expression. Transient transfection experiments showed that the AR can enhance the *Ceacam1* promoter activity in a ligand-dependent manner and that the regulatory element resides within a relatively short (−249 to −194 bp) segment of the 5′-flanking region of the *Ceacam1* gene. This androgen regulation is likely through direct AR-promoter binding because a mutant AR defective in DNA binding failed to upregulate reporter gene expression. Furthermore, electrophoretic mobility shift assays demonstrated that the AR specifically binds to this sequence, and mutation analysis of the potential ARE sequences revealed a region within the sequence that was required for the AR to activate the *Ceacam1* gene. Therefore, the regulation of *Ceacam1* gene expression by androgen may be one of the mechanisms by which androgen regulates prostatic function.

Keywords: CEACAM1; Cell adhesion molecule; Androgen receptor; Tumor suppressor; Prostate

1. Introduction

The cell–cell adhesion molecule-1 (C-CAM1), recently renamed CEACAM1 (Beauchemin et al., 1999), is a member of the immunoglobulin supergene family (Lin and Guidotti, 1989; Lin et al., 1991). CEACAM1 is mainly expressed in epithelial cells of many different tissues, including the prostate (Odin et al., 1988). Loss of CEACAM1 expression is an early event in prostate cancer progression (Kleinerman et al., 1995; Pu et al., 1999), suggesting that this molecule may play an important role in prostate tumorigenesis. Consistent with this hypothesis, expression of CEACAM1 in prostate cancer cells can suppress their tumorigenicity in vivo (Estrera et al., 1999; Hsieh et al., 1995; Luo et al., 1999). These observations suggest that CEACAM1 functions as a tumor suppressor in prostate cancer.

The prostate is an androgen-dependent organ, as androgen is the major regulator of prostate development, growth, and secretory function. Induction of prostate involution using androgen ablation is one of the most effective treatments of late-stage prostate cancer. Since CEACAM1 is a tumor suppressor in prostate cancer, it is important to know whether expression of CEACAM1 in the prostate is regulated by androgen.

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The rat *Ceacam1* promoter belongs to the GC-rich class of TATA-less promoters (Najjar et al., 1996). Deletion and substitution analyses have revealed that the three proximal Sp1 binding sites are essential for basal transcription of the *Ceacam1* gene. In addition, Najjar et al. (1996) have shown that *Ceacam1* promoter activity is stimulated 2–3-fold by insulin, dexamethasone, and cyclic adenosine monophosphate treatment. However, the effect of androgen on *Ceacam1* promoter activity has not been examined. Therefore, in this study, we examined whether the androgen receptor (AR) regulates *Ceacam1* promoter activity.

2. Materials and methods

2.1. Plasmid constructions

The 5′-flanking region of the rat *Ceacam1* gene was cloned as described previously (Najjar et al., 1996). Nucleotides were numbered relative to +1 at the ATG translation initiation codon and labeled as negative numbers to reflect their position upstream (5′) of the ATG site. Using polymerase chain reaction (PCR), 5′ deletion products (−1609, −439, −249, −194, −147, −131, −124 and −112 bp) of the *Ceacam1* gene were synthesized and subcloned at the *Xho*I and *Hin*dIII sites of the pGL3-BASIC plasmid (Promega, Madison, WI) (Najjar et al., 1996).

The mutants −249pLucARE-1Mut and −249pLucARE-2Mut were generated by site-directed mutagenesis of the −249pLuc vector using PCR. Oligo # 305 (reverse primer; AAGCTTTTCTCTTGGGGAAGA) and oligo # 306 (forward primer; *CTCGAG*ATGTTCTAGAACAATGAACCGAAAAGAGATCCCGCGAAGGATGGGAGGACAGCA) were used as primers to introduce substitutions into the ARE-1 region, while oligo # 307 (forward primer; GCTAGCCCGGG*CTCGAG*AGTCGACAGAACAATGAACCGAAAA) and oligo # 305 were used to introduce substitutions into the ARE-2 region; the sequences that were changed from the wild type are underlined. After these PCR products were sequenced to confirm the mutations, they were subcloned at the *Xho*I and *Hin*dIII sites of the pGL3-BASIC plasmid. The construction of the reporter plasmid harboring two androgen response elements and a TATA box driving the luciferase gene (p[ARE]2-E1b-luc) has been described previously (Jenster et al., 1997).

The human AR cDNA expression vector (pAR_0) was constructed using the simian virus 40 (SV40) early promoter and the rabbit β-globin polyadenylation signal as described previously (Brinkmann et al., 1989). The AR mutant expression vector pAR64, in which the first zinc finger in the AR was disrupted by the replacement of two cysteines with serine and phenylalanine, was constructed as described by Jenster et al. (1993). Additionally, the superactive AR expression vector pcDNA-AR_0p65 was constructed by inserting the *Asp718*-(filled in with the Klenow fragment) and *Sac*II digested fragment of pcDNA-AR_{LBD}-p65 into the *Hpa*I and *Sac*II digested pcDNA-AR_0mcs vector (Sui et al., 1999). This resulted in the generation of a fusion protein containing the wild-type AR fused with the transactivation domain of p65/RelA.

2.2. Culture and transfection of HeLa cells

HeLa human cervical carcinoma cells (American Type Culture Collection, Manassas, VA) were maintained in minimal essential medium supplemented with 10% (v/v) fetal calf serum (FCS). These cells (50 000) were plated in a 12-well plate with 10% (v/v) charcoal-stripped FCS 24 h before transfection. The cells were transfected with 0.3 μg of both luciferase reporter plasmid containing a *Ceacam1* promoter fragment and a receptor plasmid containing either wild-type (pAR_0) or modified AR (pAR64 or pcDNA-AR_0p65) per well using Lipofectin (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's guidelines. About 24 h after transfection, the cells were washed and fed with medium containing charcoal stripped serum with or without R1881 (17α-methyltrienolone; NEN Life Science Products, Boston, MA), and the incubations were continued for an additional 24 h. The cells were then lysed in 200 μl lysis buffer, and the luciferase activity was measured using a luciferase assay system (Promega). The experiments were performed in triplicate.

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA was carried out using a bandshift assay system (Promega). Oligonucleotides having sequences corresponding to the region between −194 to −249 bp of the *Ceacam1* promoter were synthesized by Genosys (Houston, TX) and used as probe. In addition, oligonucleotides containing the AR consensus sequence were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used as competitors. The plasmid pRSET-GST-AR_{DBD} containing a sequence from the AR DNA binding domain fused to GST was constructed by inserting 0.3 kb of the Klenow-treated *Rsr*II/*Xba*I digested ARDBD fragment from AR126 (Jenster et al., 1995) into the Klenow-treated *Nco*I/*Hin*dIII digested pRSET-GST-SRC782-1139 vector (Spencer et al., 1997). The GST-fusion protein containing the AR DNA binding domain (GST-AR_{DBD}) was expressed and purified from *Escherichia coli* BL21(λDE3), and 100 ng of GST-AR_{DBD} protein was used for EMSA. Purified GST protein was used as a negative control.

2.4. Statistic analysis

Student's t-test was used to examine R1881 effects on different types of mutation. We chose the ratio of promoter activities in the presence and absence of R1881 as a dependent variable to avoid variation in basal activity between different experiments.

3. Results

3.1. Localization of an androgen-responsive region in the Ceacam1 promoter

To examine the effect of AR on *Ceacam1* promoter activity, we first tested cell lines that express AR. Although LNCaP cells, which were isolated from the lymph node metastasis of a prostate cancer patient (Horoszewicz et al., 1983), were shown to express AR, the transfection efficiency in this cell line was very low (data not shown). Another prostatic cell line that express AR is NbE cell. NbE cell is a cell line derived from the ventral prostate of Noble rat and is shown to express AR (Chung et al., 1989). We found that the reporter plasmid containing two androgen response elements (p[ARE]$_2$-E1b-luc) could not respond to R1881 stimulation when transfected into the NbE cells (data not shown). However, this reporter was activated 90–340-folds by R1881 when it was co-transfected with a wild type AR plasmid in the NbE cells (data not shown). This observation suggested that the AR in NbE cells was not functional. The reason for AR dysfunction in NbE cells is not known. Previous studies by Jenster et al. (1995) and Sui et al. (1999) have shown that HeLa cells co-transfected with AR and promoter constructs were suitable for AR related studies. As a result, we chose to use HeLa cells co-transfected with AR for this study.

Ceacam1 promoters with different lengths that were constructed by 5′ deletion were cloned in front of the luciferase gene in the reporter plasmid. Each of these plasmids was transiently cotransfected with the AR expression vector pAR$_0$ into HeLa cells; the reporter plasmid containing two androgen response elements and a TATA box derived from the E1b gene (p[ARE]2-E1b-luc) was used as a positive control. In the absence of the androgen analogue R1881, the 1609 bp *Ceacam1* promoter mediated a 106-fold increase in reporter gene

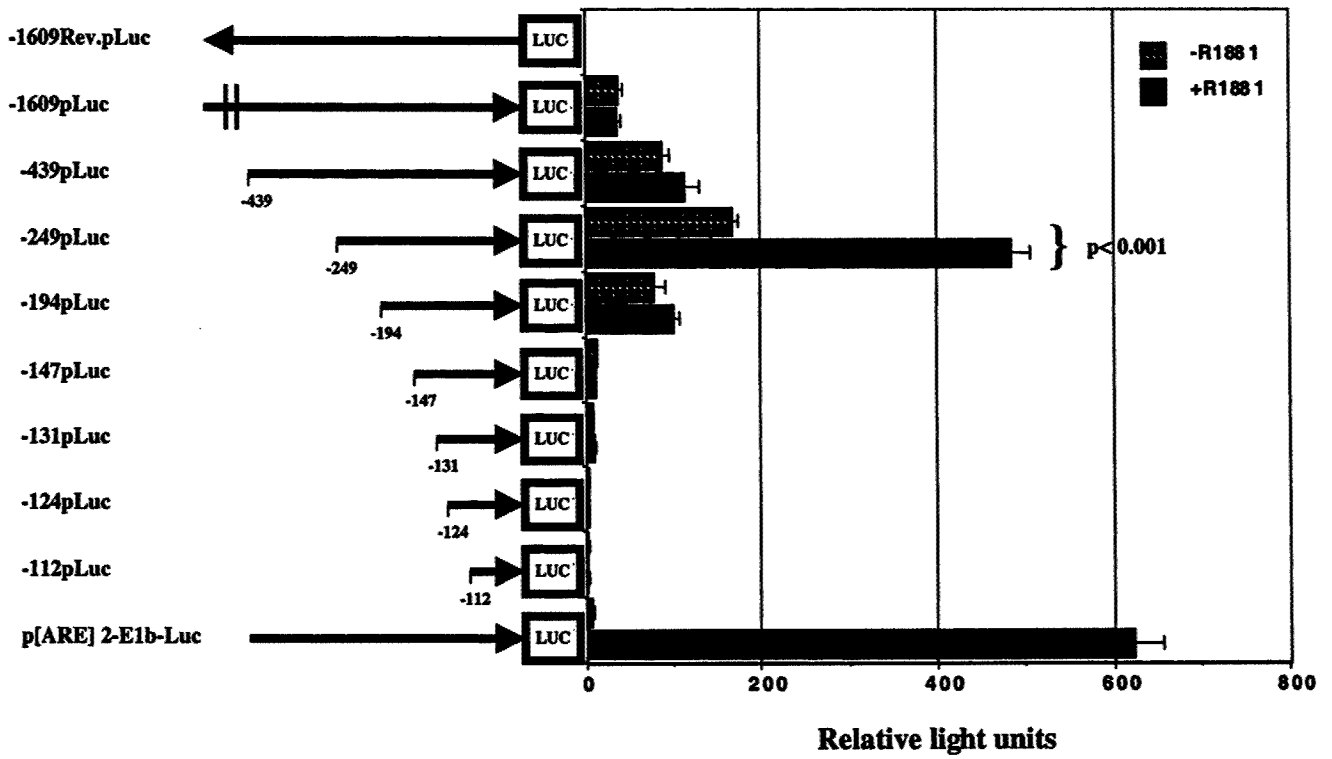

Fig. 1. Regulation of CEACAM1 expression by androgen. A series of reporter plasmids containing *Ceacam1* promoter fragments having different 5′ deletions were cotransfected with a wild-type AR plasmid (pAR$_0$) into HeLa cells. About 24 h after transfection, the cells were incubated with (+) or without (−) 1 nM R1881. The luciferase activity of these cell lysates was determined as described in Section 2. This experiment was repeated eight times with triplicate transfections for each construct and similar results were obtained. Results from one of these experiments were shown and the luciferase activities were reported as the average ± S.D. in relative light units of triplicate transfections. Statistic analysis was used to determine whether there was difference between the R1881 treated and untreated groups. Only −249pLuc construct showed statistically significant difference and the P value for −249pLuc is shown.

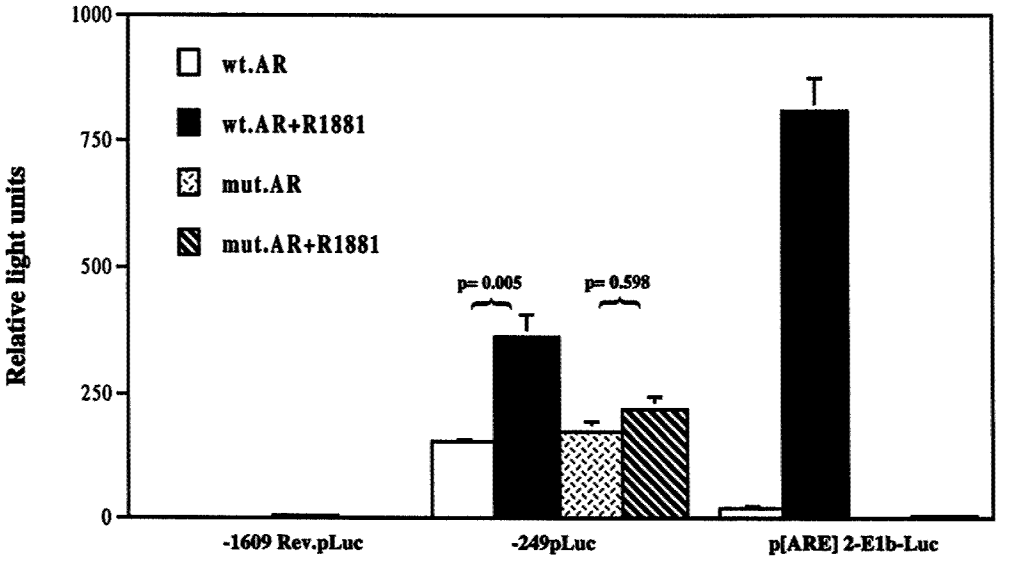

Fig. 2. Effect of an AR mutation on its ability to activate the *Ceacam1* promoter. Cells were transfected with the −249pLuc *Ceacam1* promoter together with a wild-type AR (pAR_0) or mutant AR (pAR64) plasmid, respectively. The data are presented as the mean ± S.E. of three independent experiments. Statistic analysis was used to determine whether there was difference between the R1881 treated and untreated groups. The *P* values for each group are shown.

expression as compared with the reverse-oriented *Ceacam1* promoter fragment (Fig. 1). Deletion of the region between nt −1609 and −439 induced a slight increase in the basal promoter activity (Fig. 1), suggesting that this region may contain potential down-regulators. Deletion of the *Ceacam1* promoter up to −194 bp did not abolish its ability to induce luciferase expression, while deletion up to −147 bp markedly reduced its promoter activity. This result suggested that a minimal promoter is located within the first 194 bp 5′ from *Ceacam1*'s translation start site. We next investigated whether androgen had an effect on the *Ceacam1* promoter. As shown in Fig. 1, the plasmid containing the *Ceacam1* promoter region from −249 to −21 bp exhibited a 2.5-fold increase in luciferase activity upon the addition of the androgen analogue R1881. In contrast, no significant hormone response was observed with plasmids containing the entire 1609, 439, or 194 bp segment proximal to the translation start site. These observations suggested that the region from −249 to −194 bp in the *Ceacam1* gene may contain an androgen-regulated sequence.

3.2. Direct binding of the AR to the promoter sequence

The AR is a 110–112 kDa protein containing transcriptional activation domains in its N-terminal region, a centrally located DNA binding domain, and the ligand binding domain at its C-terminus (Jenster et al., 1991). To test whether activation of the *Ceacam1* promoter by androgen is due to direct interaction between it and the AR, we investigated the effect of a mutant AR, AR64, which is defective in DNA binding (Jenster et al., 1993), on *Ceacam1* promoter activity. In contrast to the wild-type AR, AR64, when cotransfected with −249pLuc into HeLa cells, did not show significant hormone induction (Fig. 2). Similarly, p[ARE]2-E1b-Luc lost its response to R1881 stimulation. These results suggest that activation of the *Ceacam1* promoter by the wild-type AR requires its DNA binding domain; thus, AR may bind directly to *Ceacam1* promoter.

In addition, EMSA was used to determine whether the AR can bind to the promoter sequence. A double-stranded oligonucleotide containing the promoter sequence from −249 to −194 bp was used in the assay. Fig. 3 shows that the AR DNA binding domain can bind to the oligonucleotide (−249 to −194 bp) and that the binding can be specifically competed by the unlabeled corresponding oligonucleotide duplexes, as well as an unlabeled oligonucleotide containing the AR consensus sequence (Roche et al., 1992). This observation suggested that the AR binds specifically to the *Ceacam1* promoter sequence.

3.3. Identification of AR-interacting sites

Using a DNA binding site-selection assay, Roche et al. (1992) determined a consensus AR DNA binding site for the AR. Two regions in the *Ceacam1* promoter, located at −215 to −220 bp and −243 to −248 bp, respectively, showed homology to the consensus half-site sequence and could be responsible for androgen induction of the −249pLuc reporter activity (Fig. 4). These two potential AR binding sites (ARE-1 and ARE-2) were mutated to see if they are indeed involved in androgen regulation. The effect of mutating ARE-1

or ARE-2 on the promoter activity was examined. Mutations of ARE-1 did not cause a significant change in the *Ceacam1* promoter's response to R1881, while mutation of ARE-2 completely abolished the response (Fig. 4). In addition, mutating both ARE-1 and ARE-2 had a similar effect to that of mutating ARE-2 alone. These observations suggested that only ARE-2 is involved in the androgen regulation of *Ceacam1* promoter activity.

A mutational analysis of potential ARE sites was also performed using a superactive AR containing the AR fused with the transactivation domain of p65/RelA (Schmitz and Baeuerle, 1991). As part of the AR_0p65 fusion protein, the p65 activation domain can recruit additional coactivators and proteins of the preinitiation complex resulting in amplification of AR-mediated transcriptional signals. As shown in Fig. 5A, the −249 bp *Ceacam1* promoter activity showed a 5–6-fold increase in response to R1881 stimulation with the superactive AR in contrast to a 2–3-fold increase in response to R1881 stimulation with the wild-type AR. Such an enhancement of reporter activity was used to further confirm the mutational analysis. In the presence of the superactive AR, mutation of ARE-1 resulted in a 4-fold increase in luciferase activity in response to R1881. As observed with wild-type AR, R1881 treatment did not increase the promoter activity of the ARE-2 mutant or combined ARE-1/ARE-2 mutant. These observations further confirmed that the AR only requires ARE-2 to stimulate *Ceacam1* promoter activity.

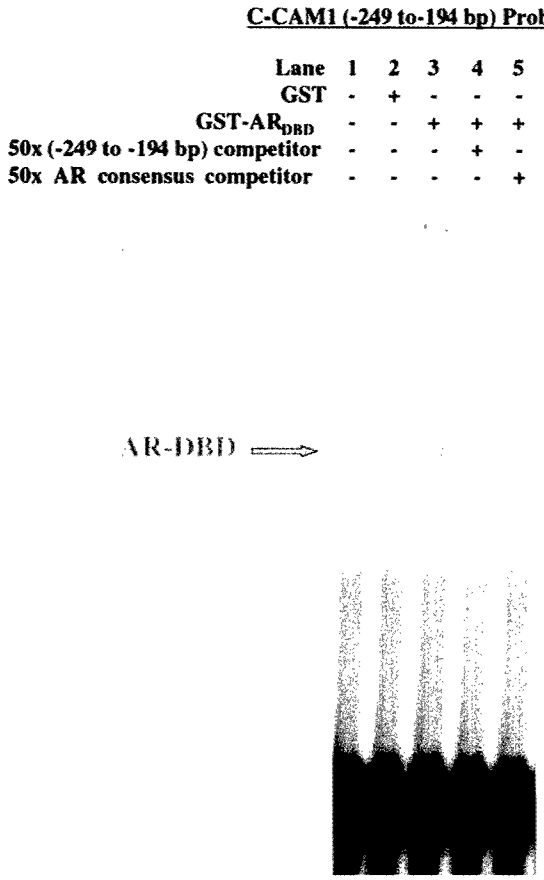

Fig. 3. EMSA, which was carried out using purified GST-AR_{DBD} and the labeled double-stranded oligonucleotide probe containing a sequence from −249 to −194 bp of the *Ceacam1* promoter. Lane 1, without protein; lane 2, with GST protein; lane 3, with GST-AR_{DBD}; lane 4, with GST-AR_{DBD} and a 50-fold molar excess of the unlabeled probe; lane 5, with GST-AR_{DBD} and a 50-fold molar excess of a double-stranded oligonucleotide containing the AR consensus sequence (Roche et al., 1992).

4. Discussion

Androgen is the most important factor that regulates prostate growth and differentiation. A series of genes that have functions related to cell-growth modulation have been shown to be regulated by androgen in prostate cells. It was shown that androgen can directly or indirectly upregulate growth factors such as epidermal growth factor (Hiramatsu et al., 1988; Nishi et al., 1996), keratinocyte growth factor (Fasciana et al., 1996; Peehl and Rubin, 1995; Rubin et al., 1995; Yan et al., 1992), and basic fibroblast growth factor (Katz et al., 1989; Zuck et al., 1992), leading to epithelial-cell proliferation. In addition, transforming growth factor β, which has been linked to programmed cell death, is induced upon androgen withdrawal (Kyprianou et al., 1990). Regulation of growth hormones and apoptotic factors may contribute to the growth of the prostate. On the other hand, androgen upregulation of insulin-like growth factor binding proteins (IGFBP) could make the potent prostate mitogens IGF-I and IGF-II unavailable for growth induction (Gregory et al., 1999). Cell-cycle regulatory proteins such as cdk2, cdk4, cyclin D3, cyclin A, p21CIP1/WAF-1, p27kip1, and p16 were also found to be regulated by androgen (Gregory et al., 1998; Knudsen et al., 1998; Kokontis et al., 1998; Lu et al., 1999, 1997). These diverse androgen-regulated events result in the maintenance of prostate homeostasis; disruption of these intricately balanced events may lead to prostate cancer initiation and progression.

In the present study, we showed that *Ceacam1*, a tumor suppressor gene, can, under defined circumstances and/or in a specific cellular context, be regulated by androgen. Specifically, androgen could up-regulate CEACAM1 expression in a ligand-dependent manner when tested in vitro. This androgen regulation is controlled by only one of the two half-sites of the AR consensus sequence (Roche et al., 1992). A similar event was also observed by Dai and Burnstein (1996), who showed that the presence of one half-site of the AR consensus sequence is sufficient to upregulate the promoter of the AR gene by the AR. This half-site interaction may not provide as strong an activity as that provided by the full consensus sequence in the

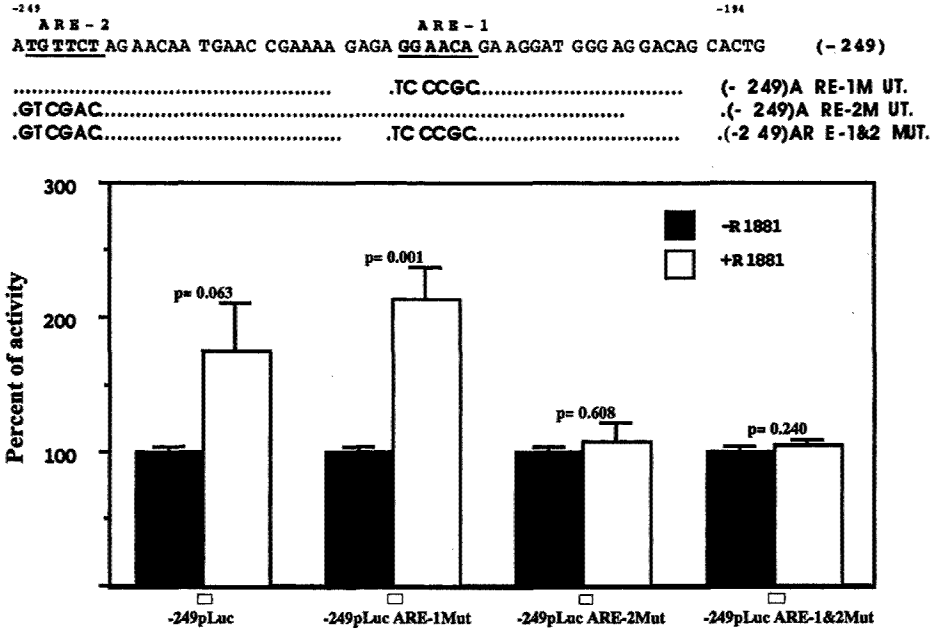

Fig. 4. Mutation analysis of the putative AREs in the *Ceacam1* promoter. The nucleotide sequence from −249 to −194 bp in the rat *Ceacam1* promoter is shown. Two half-sites of putative androgen-responsive elements (ARE-1 and ARE-2) are underlined; the mutated sequence of the two elements is shown. Cells were transfected with reporter plasmids containing the −249 bp promoters, whose putative ARE-1 or ARE-2 sequence was mutated as described in Section 2. The luciferase activity is presented as a percent of that of the corresponding plasmid containing no mutation and without R1881 treatment. The data are presented as the mean ± S.E. of three independent experiments. Statistic analysis was used to determine whether there was difference between the R1881 treated and untreated groups. The *P* values for each group are shown.

probasin promoter (Kasper et al., 1994) and PSA promoter (Zhang et al., 1997). In addition, the longer *Ceacam1* promoter, i.e. the 439-bp segment, did not show ligand-dependent regulation by androgen, suggesting that other regulatory mechanisms may be present upstream of the promoter region and may influence the interaction of the AR with the *Ceacam1* proximal promoter region. Thus, the regulation of CEACAM1 expression by the AR is complex and may depend on its cellular context. In this regard, it is interesting to note that in the rat, CEACAM1 is mainly expressed in the dorsal lobe but not the ventral lobe of the prostate, although the AR is expressed in both prostate lobes (Makarovskiy et al., 1999). In addition, using castration-induced prostate involution followed by administration of androgen, we have previously shown that expression of CEACAM1 in the rat ventral prostate was negatively regulated by androgen in vivo, while its expression in the dorsal prostate showed no response to androgen manipulation in vivo (Hsieh and Lin, 1994; Makarovskiy et al., 1999). In the mouse prostate, on the other hand, increased CEACAM1 expression was detected in the dorsal prostate lobe following castration (Pu et al., 1999). In the present study, we found that androgen could up-regulated CEACAM1 expression in a ligand-dependent manner, although up-regulation of CEACAM1 expression by androgen was observed only with a relatively short promoter (bp −294 to −197). Together, these observations suggest that the regulation of CEACAM1 expression by androgen in vivo is a complex process. Although the AR has a positive effect on the proximal region of the *Ceacam1* promoter, it may have a negative effect on the other region of the *Ceacam1* promoter. AR regulation of CEACAM1 expression is, therefore, influenced by the cellular context and possibly the stromal components of the prostate. The interactions between prostatic epithelial and stromal components have been shown to contribute to androgen-dependent regulation of prostate growth and differentiation (Chung, 1995; Cunha et al., 1987). As a result, although direct interactions between AR and *Ceacam1* promoter may contribute to androgen regulation of CEACAM1 expression, other factors including signals from stromal-epithelial interactions may also influence CEACAM1 expression in vivo.

Primary prostate cancers are largely dependent on androgens for growth and survival. Therefore, the disease responds favorably to androgen ablation and antiandrogen therapy in most patients. However, virtually all patients will relapse with clinically defined androgen-independent cancer. Previously, the role of CEACAM1 in androgen-dependent and androgen-independent prostate cancer was studied by examining the patterns of CEACAM1 expression during prostate cancer initiation, progression, and metastasis in the transgenic adenocarcinoma of mouse prostate (TRAMP) model, which was generated by using the rat probasin pro-

moter to target the SV40 large T antigen specifically to the mouse prostate (Greenberg et al., 1995). In the TRAMP mice, immunohistochemical staining using polyclonal antibody Ab669 against CEACAM1 revealed that the CEACAM1 protein was expressed in normal prostate epithelia, as well as low-grade prostate intraepithelial neoplasia (PIN); the expression was uniform on the luminal surfaces of these epithelia. CEACAM1 expression was noticeably reduced and the staining pattern was heterogeneous in some cases of high-grade PIN, and CEACAM1 staining was generally absent from prostate cancer and metastatic lymph nodes. Androgen-independent prostate cancer and its metastases generated in castrated TRAMP mice were also CEACAM1 negative (Pu et al., 1999). Since loss of CEACAM1 expression occurred before the development of androgen-independent tumors, it is likely that the AR regulation of CEACAM1 expression is not related to the loss of CEACAM1 during prostate cancer progression.

Other factors that have been shown to have an effect on the *Ceacam1* promoter include the upstream stimu-

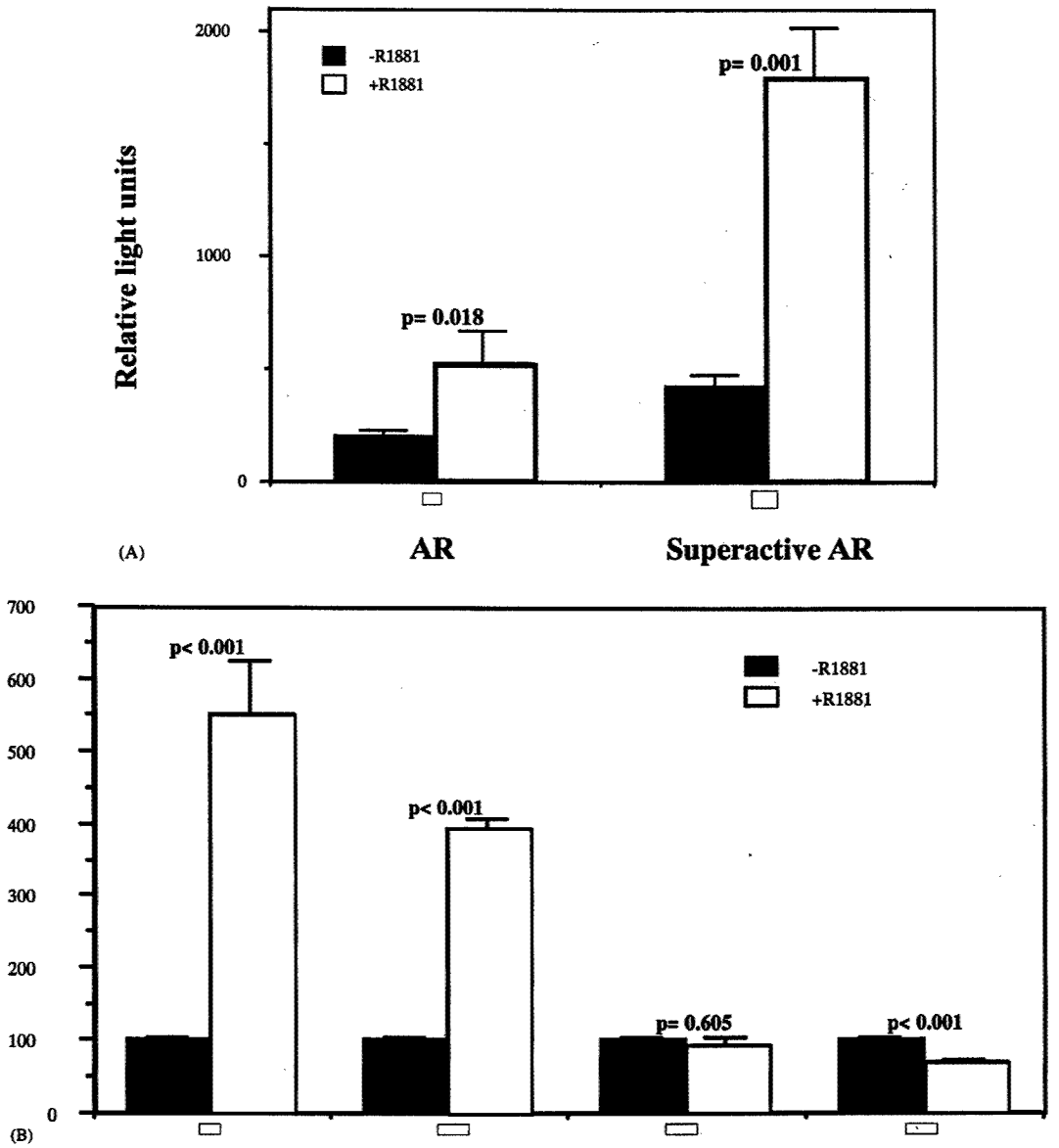

Fig. 5. Activation of *Ceacam1* promoter activity by a superactive AR (AR_0p65). (A) The *Ceacam1* promoter transcription activity was examined using cotransfection of the *Ceacam1* promoter reporter construct (− 249pLuc) and the wild-type or superactive AR expression plasmid (pAR_0p65) into HeLa cells. The luciferase activity was determined from cell lysates of transfected cells as described in Section 2. This experiment was repeated six times with triplicate transfections for each construct and similar results were obtained. Results from one of these experiments were shown and the luciferase activities were reported as the average ± S.D. in relative light units of triplicate transfections. (B) Effect of superactive AR on mutant *Ceacam1* promoter transcription activity. The luciferase activity is presented as a percent of the luciferase activity without R1881 treatment. The data are presented as the mean ± S.E. of three independent experiments. Statistic analysis were performed as described in Section 2 to compare the R1881-treated and untreated groups for each construct, and the *P* values for each group are shown.

latory factor and hepatocyte nuclear factor-4 (Hauck et al., 1994). Also, Chen et al. (1996) showed that treatment of HT-29 cells with interferon-γ (IFN-γ) upregulated CEACAM1 expression. This was due to the ability of IFN-γ to upregulate the expression of IRF-1, which, by binding to the interferon stimulated response element located in the human *Ceacam1* promoter, activated *Ceacam1* transcription. Thus, the regulation of CEACAM1 expression is a combination of different transcriptional factors, one of which is the AR.

Acknowledgements

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Tumor Specific Regulation of CEACAM1 Gene Expression by Sp2 and Histone Deacetylase

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Running title: tumor-specific CEACAM1gene regulation

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Abstract

Downregulation of CEACAM1 tumor suppressor gene expression is a common event in several malignancies including prostate, colon and breast cancer. The mechanism that leads to CEACAM1 down-regulation in tumorigenesis is not known. We found that down-regulation of CEACAM1 expression in prostate tumors is mainly due to transcriptional down-regulation of CEACAM1 gene. In addition, we provide evidence that down-regulation of CEACAM1 gene is mediated by Sp2, which is highly expressed in prostate cancer cells. Inhibitors of histone deacetylase dramatically potentiate CEACAM1 expression in prostate cancer cell lines suggesting that Sp2 inhibits CEACAM1 gene expression through recruitment of histone deacetylase. Thus, loss of CEACAM1 tumor suppressor gene expression in prostate cancer is due to aberrant chromatin acetylation.

Introduction

Lost of tumor suppressor is one of the major mechanisms that lead to tumor formation. Although tumor suppressors were originally identified as genes whose deletions or mutations caused tumor, down-regulation of proteins is a more general phenomenon in tumorigenesis (1). Sager et al. (1) thus defined the former as type I and the later as type II tumor suppressors. The type II tumor suppressor constitutes the major group of genes whose loss of expression resulted in tumorigenesis.

CEACAM1 is a 105 kDa glycoprotein originally identified as a protein that mediated intercellular adhesion (2). Down-regulation of CEACAM1 was observed in many tumor types including prostate (3, 4), colon (5-7), endometrin (8), breast (9, 10), and hepatocellular (11) carcinomas suggesting that CEACAM1 may have an important role in the maintenance of normal epithelial phenotype. In experimental tumor models, reduced expression of CEACAM1 in NbE cells, generated from normal rat prostate, promotes tumorigenicity (12) and re-expression of CEACAM1 in prostate cancer cells suppressed their tumorigenicity in vivo (12). Suppression of tumorigenicity by CEACAM1 was also observed with breast (13), bladder (14), and colon carcinoma (15). In addition, the human (16), rat (12), and mouse homologues of CEACAM1 (15) were all shown to have tumor-suppressive activity. These results support the role of CEACAM1 as a tumor suppressor.

Inhibition of tumor angiogenesis may be involved in CEACAM1-mediated tumor suppression. Volpert et al. (17) showed that conditioned medium from CEACAM1-expressing cells inhibited endothelial cell migration *in vitro* and corneal angiogenesis *in vivo*. This effect is specific to endothelial cells and not to epithelial cells (17). Thus, it is likely that expression of CEACAM1 in tumor cells induces the production of an inhibitory factor or factors that affect tumor angiogenesis, leading to suppression of the tumor growth *in vivo*. These observations

suggest that CEACAM1 plays a critical role in pathways that regulate the progression of prostate cancer.

The mechanism by which CEACAM1 is lost during tumorigenesis is not clear. Allelic loss of CEACAM1 gene, localized at chromosome 19 (18, 19) and 7 (20) in human and mouse, respectively, has not been reported for either prostate or colon cancer. Although extensive analysis on human prostate cancer specimen has not been performed, studies by Rosenberg et al., (21) using tissues or cell lines of mouse colon carcinoma showed that neither chromosomal rearrangements nor gene deletions occurred close to the CEACAM1 genes. Thus, it is likely that down-regulation instead of irreversible loss of CEACAM1 expression in vivo is the major cause of tumorigenesis.

In this study, we identify the mechanism of loss of CEACAM1 expression in prostate cancer. We found that down-regulation of CEACAM1 expression in prostate tumors is mainly due to transcriptional down-regulation of CEACAM1 gene. In addition, we provide evidence that down-regulation of CEACAM1 gene is mediated by Sp2, which is highly expressed in prostate cancer cells, and Sp2 recruits histone deacetylase to repress transcription of CEACAM1 gene. Thus, loss of CEACAM1 tumor suppressor gene expression in prostate cancer is due to aberrant chromatin acetylation.

Experimental Procedures

Western and Northern Blot Analyses

For Western analysis, the cells were trypsinized into a single-cell suspension and lysed in modified RIPA buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 1% NP-40, 0.5% deoxycholate, 1 mM PMSF, 1 mM sodium vanadate, protease inhibitor tablet (Roche)). The protein concentrations of the cell lysates were determined by Coomassie Blue Plus (Pierce Chem. Comp. Rockford,

Illinois). Equal amounts of protein from each lysate were boiled in SDS sample buffer, and Western blot analysis was performed as described previously (22) with anti-CEACAM antibody Ab669. For northern analysis, total cellular RNA was extracted from cells by using RNAzolB (Biotecx Laboratories, Inc., Houston, TX) according to manufacturer's instructions. Twenty ug of RNA was subjected to northern blot analysis by electrophoresis on a 1% agarose gel containing 0.02% formaldehyde as described (23), and the blot was hybridized with a CEACAM1 cDNA probe (24).

CEACAM1 Promoter Plasmid Construction

The 5'-flanking region of rat CEACAM1 gene was cloned as previously described (25). Using PCR, 5' deletion products (-1609bp, -439bp, -249bp, -194bp) of the CEACAM1 gene were synthesized and subcloned at the XhoI and HindIII sites of pGL3-BASIC plasmid (25). The mutant p-194mutLuc was generated by site-directed mutagenesis of the p-194Luc vector using PCR. Oligo 309 (forward primer; 5' CTCGAGTGAGAGAACAGCATTGTCAGAAATTACTTTACCACCCCCCAGCCCA 3') and Oligo 304 (reverse primer; 5' AAGCTTCTTCTCTTGGGGAAGAGAT 3') were used as primers to introduce substitutions into the putative Sp2 binding region. The sequences that were changed from the wild type are underlined. The PCR product was subcloned into TOPO-PCR2.1 vector (Invitrogen, Carlsbad, California) and sequenced to confirm the mutations. The PCR product was subcloned at the XhoI and HindIII sites of the pGL-BASIC plasmid to generate plasmid p-194mutLuc.

Transfection of NbE and Mat-LyLu cells

NbE cells (26) were maintained in DMEM medium supplemented with 10% fetal calf serum. Mat-LyLu cells (27) were cultured similarly except that 250 nM dexamethason was included in the medium. These cells (50,000) were plated in a 12-well plate 24 h before

transfection. The cells were transfected with 0.3 ug of plasmid per well using Lipofectamine (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's guidelines. Twenty-four hours after transfection, the cells were lysed in 200 ul lysis buffer and the luciferase activity was measured using a luciferase assay system (Promega, Madison, Wisconsin). For the experiments in which cells that were treated with TSA, TSA (1 ug/ml) was added to the medium five hours after the cells were transfected with plasmid. The cells were incubated with TSA for 24 hr and luciferase activity was determined. All experiments were performed in triplicate.

Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts was performed as described (28). Protein concentrations were determined by using the Coomassie blue Plus protein assay reagent kit (Pierce, Rockford, IL) according to the manufacturer's procedures and 10 ug of nuclear protein was subjected to electrophoretic mobility shift assays (EMSA). EMSA was carried out using a bandshift assay system (Promega). Double-stranded oligonucleotides with sequences corresponding to the region between -147 to -194 bp (194 probe) or 194 probe containing mutation (194mut probe) of CEACAM1 promoter were synthesized by Sigma/Genosys (Houston, TX) and used as probe. For supershift analyses, nuclear extracts were incubated with 1 µl rabbit anti-human polyclonal antibody specific for Sp1, Sp2, Sp3, Sp4 (Santa Cruz Biotechnology).

Immunostaining

Cells were plated overnight to allow them to attach. Cells were washed in PBS, fixed with formalin, blocked with normal goat serum at room temperature for 30 minutes, and incubated at 4°C overnight with Sp2 (K-20) antibody (Santa Cruz). The antibody binding was detected by using ABC kit (Vector laboratory, Burlingame, CA) according to the manufacturer's instructions with 3,3'-diaminobenzidine as the chromogen. The immunostained sections were then counterstained with hematoxylin.

Chromatin immunoprecipitation (ChIP) assays -- Quantitative ChIP assays were carried out as described (29, 30) using the ChIP assay kit (UBI). NbE or Mat-LyLu cells grown on 10 cm culture dishes were treated with formaldehyde to a final concentration of 1% at 37°C for 10 min. The cells were scraped from the plates in phosphate-buffered saline (PBS) containing protease inhibitors. The cell pellets were resuspended in lysis buffer and sonicated to reduce DNA size to 200 to 1000 bp. The cell debris was removed by centrifugation and the supernatant that contained the sheared chromatin were saved for further analysis. Aliquots of these chromatin preparations are used to determine the total DNA content and for normalizing the PCR signals. The remaining chromatin solution is used for immunoprecipitation. Anti-acetyl histone H4 (Anti-AcH4) antibody (#06-866, Upstate Biotechnology) or Sp2 antibody (K-20, Santa Cruz) was added to chromatin solution and incubate overnight. The immune complexes were collected with protein-A agarose. The protein-A agarose beads were pelleted by centrifugation and the immune complexes eluted by elution buffer. The crosslinked protein-DNA complexes were incubated at 65°C for 4 hours to reverse the crosslink. The proteins were digested with proteinase K and DNA recovered by phenol/chloroform extraction and ethanol precipitation.

PCR using primer pairs corresponding to –162 to –240 region of the CEACAM1 promoter was used to detect the CEACAM1 promoter that forms complex with either acetylated histone H4 or Sp2. Histone 3.3 was used as a control for total chromatin input. The primers for H3.3 were: forward 5'-GCAAGAGTGCGCCCTCTACTG-3' and reverse 5'-GGCCTCACTTGCCTCCTGCAA-3'. Data are representative of four ChIP assays using anti-acetylated histone H4 antibody and two ChIP assays using anti-Sp2 antibody. Real Time PCR was used to confirm the relative differences between ChIP samples. The primers and probe for detecting CEACAM1 promoter were designed by using TaqMan 7700 sequence detection system (Perkin Elmer Life Sciences). For promoter region from –162 to –240, the primers used in Real time PCR are: forward primer AACAATGAACCGAAAAGAGAGGAA (nt -240 to –217),

reverse primer GAGCCTGCGACTCTGACAATG (nt –183 to –163), and the Tag-Man probe GTTCTCTCAGTGCTGTCCTCCCATCCTTCT (-215 to –186). Values were normalized to CEACAM1 promoter DNA levels in total sheared DNA samples.

Results

CEACAM1 protein in normal and prostate carcinoma cell lines

To study the regulation of CEACAM1 gene during prostate tumorigenesis, we first characterized CEACAM1 expression in the Dunning rat prostate cancer cell lines to determine if CEACAM1 protein expression levels show distinct tumor-specific down-regulation. The Dunning 3327 prostate cell line was isolated from a rat prostate tumor by Dunning (31) from a 22-month-old inbred Copenhagen male rat. Following serial in vivo passage of the original R3327 tumor, sublines (Dunning H, Dunning G, AT-1, AT-2, AT-3, Mat-Lu, Mat-LyLu) with different biological characteristics were obtained and characterized (27). These cell lines represent tumors ranging from relatively benign, slowly growing, differentiated, androgen-sensitive tumors to rapidly growing, anaplastic, hormone-insensitive malignant tumors. We characterized CEACAM1 expression in AT-2, AT-3, Mat-LyLu of Dunning cell lines. The levels of CEACAM1 protein expression in these rat prostatic cancer cell lines were examined by western immunoblot analysis. A prostate cell line NbE, derived from ventral prostate of Noble rat (26), was used as a control. As shown in Fig. 1A, a significant decrease in CEACAM1 protein levels occurred in the rapid growing tumors, i.e. AT-2, AT-3 and Mat-LyLu. In Mat-LyLu cell line, CEACAM1 expression level is about 4% as compared to that of normal control cell line NbE. These results are consistent with our previous observation that CEACAM1 is down-regulated in the human prostate cancer cell lines (16) and the immunohistochemical study of mouse prostate cancer tissues, which showed loss of CEACAM1 expression in prostate carcinoma (3). In addition, we have previously shown that NbE cells, which express CEACAM1, were not tumorigenic when injected into nude mice. However, down-regulation of CEACAM1 level by transfection of anti-

sense CEACAM1 cDNA rendered NbE cells tumorigenic when injected into nude mice (12). Taken together, these observations support that down-regulation of CEACAM1 is associated with prostate cancer progression. NbE cells, which express CEACAM1 protein, and Mat-LyLu cell line, which has lowest CEACAM1 level, were chosen for further studies.

CEACAM1 Messages in Prostate Cancer Cell Lines

Since decrease in protein expression can arise from transcriptional and post-transcriptional events, we further examine the levels of CEACAM1 messages in the NbE and Mat-LyLu cell lines. Using a probe generated from full-length CEACAM1 cDNA (24), Northern blotting showed a significant decrease in the steady-state levels of 4 kilobase (kb) mRNA for CEACAM1 in Mat-LyLu cells (Fig. 1B). These observations indicate that loss of CEACAM1 protein is due to the reduction of CEACAM1 transcript. Thus, down-regulation of CEACAM1 expression in prostatic cancer cells could be due to altered transcriptional activity, which could result from changes in the *cis* elements, e.g., methylation, in the CEACAM1 promoter or the *trans* factors, e.g., transcription factors, which regulate promoter activity.

Promoter Methylation

Aberrant hypermethylation of 5' CpG islands within proximal promoter regions has been implicated as a mechanism by which tumor suppressor genes can be inactivated. Examples of this mechanism have been best demonstrated for the VHL and p16 tumor suppressor genes (32, 33). In addition, Graff et al. (34) have also shown that hypermethylation is one of the mechanisms for the down-regulation of the "metastasis suppressor" gene, i.e., E-cadherin, in prostate cancer. Genomic clone containing the CEACAM1 promoter has been isolated (25). Promoter sequence analysis showed that CEACAM1 promoter does not have high CpG content typical for genes whose expressions were modulated by methylation. Only 5 CpG dinucleotides were found within the 250 bp promoter proximal region of CEACAM1 promoter. Treatment of

the Mat-LyLu cells with DNA methylation inhibitor 5-aza-2'-deoxycytidine (35) did not result in an increase of CEACAM1 protein expression as judged by RT-PCR and Western blot analysis (data not shown). Thus, promoter methylation was unlikely to be involved in CEACAM1 regulation. This observation is also consistent with the report by Rosenberg et al. (21) that methylation was not detected in mouse CEACAM1 gene in colon carcinoma samples examined.

Transcriptional regulation of CEACAM1 gene expression in prostatic cancer cell lines

Since CEACAM1 down regulation seems to occur at the transcriptional level, we next examine how transcriptional regulation of CEACAM1 gene occurs in tumorigenesis. We have previously isolated a genomic CEACAM1 gene (25). The promoter region of this gene (1.6 kb) was cloned in front of a luciferase reporter gene and a series of transfection studies were performed to localize the promoter sequence. Using promoter deletion analysis, the minimal promoter is located between -194 and -147 bp 5' of the CEACAM1 translation start site (25, 36).

In order to compare promoter activities in the CEACAM1 positive and negative cell lines, we transfected the CEACAM1 promoter-luciferase constructs into NbE and Mat-LyLu cells. Since the tumor-specific regulatory elements may reside outside the minimal promoter, we tested the activity of -1609 bp, -439 bp, -249 bp and -194 bp promoter constructs. The transfection efficiencies were normalized against the Renilla luciferase activity from pTK-Ren, in which the Renilla luciferase expression was driven by a thymidine kinase promoter. As shown in Fig. 2, a similar pattern of promoter activity was observed in the four promoter constructs tested in the Mat-LyLu cells as compared with the NbE cells. However, there is a significant decrease, about 4-fold, in the overall CEACAM1 promoter activity in Mat-LyLu cells. These results suggest that down-regulation of CEACAM1 in prostatic cancer cells may be due to altered transcription factors that regulate CEACAM1 promoter activity and that one of the tumor-specific promoter regulatory activities lies between -194 and -147 bp in the minimal CEACAM1 promoter region.

Binding of NbE and Mat-LyLu nuclear extract to CEACAM1 promoter

Because loss of transcriptional activator(s) or activation of transcriptional repressor(s) could be one of the mechanisms responsible for CEACAM1 down-regulation in tumorigenesis, gel shift assays were used to examine the binding of nuclear proteins from NbE or Mat-LyLu cells to a double stranded oligonucleotide containing the minimum promoter sequence (nt -194 to -147, 194-probe) of the CEACAM1 promoter. As shown in Fig. 3A, four major bands (complex I-IV), were detected in the Mat-LyLu nuclear extract. The binding of complex I-IV is specific as they can be competed by excess of unlabeled 194probe. In contrast, three major bands (complex II to IV), which can be competed by excess of unlabeled 194probe, were detected in the NbE nuclear extract (Fig. 3A). Because complex I is only present in the Mat-LyLu extract, we investigated the possibility that the protein(s) present in complex I may be related to the decreased CEACAM1 promoter activity in Mat-LyLu cells.

Involvement of Sp transcription factor family at the CEACAM1 promoter

Sequence analysis of 194-probe indicates that this region of CEACAM1 promoter is highly GC-rich and it contains elements that match the consensus sequence for Sp1 binding site (25). Thus, it is likely that these complexes contain Sp family of transcription factors. EMSA supershift using antibody against the Sp1, Sp2, Sp3, and Sp4 was used to identify the protein(s) present in complex I-IV in Mat-LyLu cells. As shown in Fig. 3B, complex I, which is present in nuclear extracts from Mat-LyLu but not NbE cells, was shifted by anti-Sp2 antibody as judged by the decrease of complex I intensity (Fig. 3B, lane 5). Complex II was shifted by anti-Sp3 antibody (Fig. 3B, lane 6). Anti-Sp1 antibody generated a weak supershifted band, however, no significant decrease was observed in any of the complexes, suggesting Sp1 might be a minor component in these complexes. It is very likely Sp1 is associated with complex II as it was reported that Sp1 and Sp3 competed for the same binding sites in vivo (37). Anti-Sp4 antibody

did not generate supershifted band and no decrease of complex intensity was detected. Since Sp2 appears to be the major protein in complex I, it is likely that Sp2 is one of the transcription factors that suppress CEACAM1 promoter activity in Mat-LyLu cells.

Levels of Sp2 protein in cells correlate with CEACAM1 down-regulation

Down-regulation of CEACAM1 in Mat-LyLu cells might be due to an increase in the nuclear protein concentrations of Sp2 in Mat-LyLu (CEACAM1 low-expressing) as compared with NbE (CEACAM-1 high-expressing) cells. Western blot analysis was performed on cell extracts derived from Mat-LyLu or NbE cells using antibodies against Sp2. As shown in Fig. 4A, protein levels of Sp2 was 10-fold higher in Mat-LyLu cells than NbE cells.

Immuno-localization of Sp2 showed both nuclear and cytoplasmic staining of Sp2 in Mat-LyLu and NbE cells, however, stronger signals were obtained from nuclear staining suggesting that they are mainly localized in the nucleus (Fig. 4B). Consistent with western blot analysis, the amount of Sp2 protein is higher in Mat-LyLu cells compared with that of NbE cells by both immunofluorescence (not shown) and immunoperoxidase detection using DAB (Fig. 4B). Thus, the relative levels of Sp2 in Mat-LyLu and NbE cells correlate with potential repressive functions of Sp2 in down-regulating the CEACAM1 promoter in these cells.

Suppression of CEACAM1 expression by Sp2

To investigate whether Sp2 has a direct effect on the CEACAM1 promoter activity, we co-transfected Sp2 expression construct with CEACAM1 promoter constructs into NbE cells, which have low levels of Sp2. Overexpression of Sp2 resulted in inhibition of the CEACAM1 promoter activity (Fig. 5A), suggesting that Sp2 is a transcription repressor of CEACAM1 promoter.

To investigate whether Sp2 has an effect on the expression of the CEACAM1 protein in the cells, we transfected Sp2 expression vector into NbE cells. Overexpression of Sp2 resulted in suppression of the levels of CEACAM1 protein in NbE cells (Fig. 5B). This observation indicates that Sp2 has an effect on the expression of endogenous CEACAM1 protein suggesting that Sp2 is one of the factors that cause decreased expression of CEACAM1 protein in Mat-LyLu cells. Taken together, these results indicate that increased expression of Sp2 in Mat-LyLu cells suppresses the expression of CEACAM1.

Sp2 binding sequence at the CEACAM1 promoter

Sp-family transcription factors were known to bind to G-rich elements such as the GC-box and the related GT-box (37). Sp2 was cloned because it binds in vitro to a GT-box promoter element within the T-cell antigen receptor a gene (38). The 194 probe contains a GT-box like sequence between nt-172 to -163 and thus, this GT-box like sequence may be the potential Sp2 binding sequence. These nucleotides were mutated to generate a 194-mut probe and used as a competitor in EMSA. Addition of 100-fold excess of the 194-mut probe blocked complex II-IV formation, but did not alter complex I formation (Fig. 3, lane 8). Because the 194-mut probe cannot compete for Sp2 binding to complex I, this observation suggests that Sp2 may bind to the GT-rich sequence between nt-172 to -163 in the CEACAM1 promoter.

Mutation of Sp2 binding site increases promoter activity

Because mutation of nt -172 to -163 of CEACAM1 promoter resulted in a loss of Sp2 binding, a -194pLuc reporter containing the mutation of the Sp2 site (-194mutpLuc) was used to determine if loss of Sp2 binding results in an increase in CEACAM1 promoter activity. The -194mutpLuc reporter construct was transfected into Mat-LyLu cells and its promoter activity compared with that of wild type -194pLuc reporter construct. Mutation of the Sp2 binding site

reproducibly resulted in a 6 to 7-fold increase in CEACAM1 promoter activity in Mat-LyLu cells compared to that of wild-type promoter (Fig. 5C), suggesting that binding of Sp2 to nt -172 to –163 of CEACAM1 promoter leads to suppression of CEACAM1 promoter activity in prostate cancer cells.

Association of Sp2 with CEACAM1 promoter in vivo

We further investigated whether Sp2 is associated with the CEACAM1 promoter in vivo by using a chromatin-immunoprecipitation (ChIP) assay. Mat-LyLu or NbE cells were incubated briefly with formaldehyde to crosslink proteins to DNA. Following sonication, chromatin was immunoprecipitated with anti-Sp2 antibody to examine its specific association with the CEACAM1 promoter. The genomic DNA fragments bound to Sp2 were analyzed by PCR using primers corresponding to nt –162 to –240 region of the CEACAM1 promoter. The association of Sp2 with CEACAM1 promoter was higher in the Mat-LyLu cells compared to that of NbE cells (Fig. 5D). Quantitative PCR using primers spanning the nt –162 to –240 of CEACAM1 promoter detected a 2.7-fold increase of CEACAM1 promoter association with Sp2. These results suggest that there is an increased association of Sp2 with endogenous CEACAM1 promoter in Mat-LyLu cells compared to NbE cells.

Inhibition of HDAC activity by TSA activates CEACAM1 promoter activity

The conformation of genes within chromatin determines whether a gene is in its active or inactive state. These structural features are regulated by enzymes that modify chromatin structure. Histone acetylation leads to open chromatin conformation that promotes gene transcription by making promoter sequences accessible to transcription factors. Association with histone deacetylase (HDAC) contributes to the suppressive activity of several transcription factors (39). To investigate whether the inhibitory effect of Sp2 on CEACAM1 promoter activity in Mat-LyLu cells was mediated by HDAC, the HDAC inhibitor trichostatin A (TSA) (40) was

used to examine if it can relieve Sp2-mediated repression at the CEACAM1 promoter. Mat-LyLu cells were transfected with -194pLuc or -194mutpLuc and treated with or without TSA. Treatment of cells with TSA resulted in about 19-fold increase of -194pLuc activity while TSA did not have an effect on -194mutpLuc activity (Fig. 6A). These results suggest that Sp2 represses transcription of CEACAM1 gene by recruitment of histone deacetylase.

Acetylation state of CEACAM1 promoter in vivo

To address whether histone deacetylases might be involved in transcriptional down-regulation of the CEACAM1 gene in prostate cancer cells, the association of acetylated histone H4 (AcH4) with the CEACAM1 promoter in NbE and Mat-LyLu cells were analyzed by chromatin immunoprecipitation (ChIP) assay using anti-AcH4 antibody. The genomic DNA fragments bound to anti-AcH4 antibody were analyzed by PCR using primer pairs corresponding to nt –162 to –240 of the CEACAM1 promoter. The amount of the CEACAM1 promoters associated with anti-AcH4 antibody was higher in the NbE cells compared to that of Mat-LyLu cells (Fig. 6B). Quantitative PCR using primers spanning the nt –162 to –240 of CEACAM1 promoter showed that the amount CEACAM1 promoter associated with anti-AcH4 antibody in the NbE cells is 1.7-fold compared to that of Mat-LyLu cells. These results suggest that CEACAM1 promoter region is in more "acetylated" and thus in more "active" state in NbE cells compared to that of Mat-LyLu cells.

Taken together, these observations suggest that association of Sp2 with HDAC leads to decreased chromatin acetylation of CEACAM1 promoter and this results in decreased CEACAM1 promoter activity in Mat-LyLu cells.

Discussion

We studied the mechanisms that down-regulate CEACAM1 expression in prostate cancer. Several lines of evidence indicate that Sp2 may be involved in the down-regulation of

CEACAM1 in prostate tumors. First, Sp2 bound to CEACAM1 promoter; Second, Mat-LyLu prostate cancer cells, which is tumorigenic when injected into nude mice, but not the non-tumorigenic NbE prostate cells, expressed high level of Sp2. Third, overexpression of Sp2 in NbE cells inhibited the CEACAM1 promoter activity and also the endogenous level of CEACAM1 protein. Fourth, mutation of the putative Sp2 binding site within the CEACAM1 promoter increased the CEACAM1 promoter activity. Finally, Sp2 was shown to associate with CEACAM1 promoter in vivo in Mat-LyLu cells. These observations suggest that transcriptional suppression of CEACAM1 gene by Sp2 is one of the mechanisms that down-regulate CEACAM1 expression in prostate cancer. In addition, we showed that Sp2 repress CEACAM1 transcription through recruitment of HDAC because treatment of Mat-LyLu cells with HDAC inhibitor activated CEACAM1 promoter activity and the CEACAM1 promoter was more acetylated in NbE cells compared to that of Mat-LyLu cells.

Sp2 is the first transcription factor identified that suppresses CEACAM1 transcription. The transcription factor Sp1 was originally identified as a general transcription factor that binds to the GC-boxes and regulated many ubiquitous genes (37). Molecular cloning revealed that Sp1 is one of the members of gene family, Sp-family, transcription factors. Currently, Sp family consists of at least five proteins designated Sp1 through 5 (37, 41). Extensive studies have been reported for the function of Sp1 and Sp3 (37) and function of Sp4 has been demonstrated in several systems (37). In contrast, very little is known about Sp2 and the early reported study on Sp2 showed that it binds to a GT-box promoter element within the TCRα promoter in vitro (38). A recent study by Bakovic et al. (42) showed that Sp2 represses Sp1- and Sp3-driven transcription of CTP:phosphocholine cytidylyltransferase α promoter in Drosophila SL2 cells. Sp2 was shown to express in Jurkat T-cell line, the BJA-BB-cell line, K562 erythroid cells, and HeLa cells (38) and thus, Sp2 was considered to be ubiquitously expressed. However, the expression of Sp2 in normal tissues versus tumor cell lines was not extensively examined. Our study suggests that expression of Sp2 may be related to tumorigenesis. Thus, high level of Sp2

in these cell lines, which are generated from tumors, may be related to increased expression of Sp2 during tumorigenesis. Further study will be needed to confirm this hypothesis. Our study shows that Sp2 plays an important role in the regulation of CEACAM1 gene expression in tumorigenesis. This observation raises interesting issues concerning the genes that are regulated by Sp2 and the role of Sp2 in regulating cell growth and differentiation. Further study on the function of Sp2 is warranted.

Chromatin remodeling is a fundamental mechanism governing gene regulation during embryonic development and it may play a role in tumorigenesis. Histone deacetylase has an important role in these processes (43). For example, histone deacetylase was shown to involve in the development of acute promyelocytic leukemia (APL), and inhibitors of histone deacetylase can restore retinoid responses of RA-resistant APL cell lines (44, 45). At least nine HDACs, which can be classified to class I or II HDACs, have been identified so far in mammalian systems (46, 47). Transcription repression through HDACs can arise from a direct interaction of transcription factors with HDACs or indirectly by co-repressors or adaptors. Transcriptional repression by YYI was mediated by direct interaction with HDAC2 (48) while Mad associate with HDAC2 indirectly through a transcriptional corepressor mammalian Sin3 (mSin3) (49). Our observations that CEACAM1 promoter activity was increased by TSA treatment and the CEACAM1 gene promoter was hypoacetylated in cancer compared to normal cells by ChIP assay suggest that histone deacetylation is the biochemical basis of down-regulation of CEACAM1 gene expression in tumorigenesis. However, we do not have evidence that Sp2 binds to HDAC1 directly by co-immunoprecipitation assay (data not shown). We cannot rule out the possibility that Sp2 may interact with other members of HDAC family. In addition, Sp2 may recruit HDACs indirectly through co-repressors or other adaptor proteins. These possibilities will require further investigation.

This work reported here demonstrates that HDAC, which is tethered to CEACAM1 promoter by Sp2, deacetylates proximal histones, leading to an altered chromatin structure that

prevents transcriptional initiation of CEACAM1 gene. Thus, Sp2 mediates deacetylation of histones within chromatin. Thus, histone deacetylation is one of the regulatory mechanisms governing CEACAM1 tumor suppressor gene expression and support the notion of a close connection between HDACs and cancer.

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Figure Legends

Fig. 1. Down-regulation of CEACAM1 in prostate cancer cells. (A) Immunoblot analysis of CEACAM1 protein expression in NbE and Dunning rat prostate cancer cell lines, using polyclonal anti-CEACAM1 antibody (Ab669) (50). The blot was reprobed with anti-actin antibody as a loading control. The intensity of signal from CEACAM1 protein was quantitated by Quantity1 (BioRad). In the Mat-LyLu cell line, the CEACAM1 expression level is about 4% compared with that of normal NbE cells. (B) Northern blot analysis of CEACAM1 expression. The levels of CEACAM1 messages in the NbE, AT2, AT3.1, and Mat-LyLu cell lines were tested by Northern blot analysis using a probe generated from the full-length CEACAM1 cDNA (24).

Fig. 2. Comparison of CEACAM1 promoter activities in NbE and Mat-LyLu cells. A series of reporter plasmids containing various lengths of the CEACAM1 promoter were transfected into NbE or Mat-LyLu cells as described in Materials and Methods. CEACAM1 promoter (-1600 bp) cloned in reverse orientation (p-1609revLuc) was used as a reference. NbE or Mat-LyLu cells transfected with pTK-Renilla were used to normalize the transfection efficiency. After normalization by renilla activities, the average luciferase activities ± S.D. of triplicate transfections were shown. Values located at each construct indicate the fold difference in the promoter activity in NbE cells compared with that observed for Mat-LyLu cells. The experiment was repeated three times with triplicate transfections for each construct and similar results were obtained. Results from one of the experiments were shown.

Fig. 3. (A) Electromobility shift assay of the interaction between NbE or Mat-LyLu nuclear extract with 194probe. Oligonucleotides having sequences corresponding to the region between -194 to -147 bp of the CEACAM1 promoter were synthesized and used as probe (194probe). Nuclear

extracts from the NbE or Mat-LyLu were used. Positions of shifted complexes (complexes I to IV) were indicated by arrows. (B) Electromobility shift assay in the presence of SP-1, SP-2, SP-3, and SP-4 antibodies. Gel shift analysis using 194-probe was performed in the absence or presence of antibodies against Sp1, Sp2, Sp3, or Sp4 (Lanes 4-7). Lane 4, anti-Sp1 antibody generated a weak supershifted band, however, no significant decrease was observed in any of the complexes, suggesting Sp1 might be a minor component in these complexes. Lane 5, complex I was shifted by anti-Sp2 antibody as judged by the decrease of complex I intensity. Lane 6, complex II was shifted by anti-Sp3 antibody. Lane 8, gel shift assay in the presence of 100-fold molar excess of the 194mut probe as competitor.

Fig. 4. (A) Differential expression of Sp2 in NbE versus Mat-LyLu cells. Cell lysates (20 ug) prepared from NbE or Mat-LyLu cells were boiled in SDS sample buffer and the proteins were separated by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane and blotted with anti-Sp2 antibodies. (B) Localization of SP-2 in Mat-LyLu and NbE cells. Mat-LyLu or NbE cells grown on coverslips were fixed by formaldehyde and immunostained with antibody against Sp2. The localization of Sp2 protein was detected by diaminobezidine.

Fig. 5. Suppression of CEACAM1 expression by Sp2. (A) Sp2 decreases transcriptional activity of CEACAM1 promoter in NbE cells. NbE cells were co-transfected with CEACAM1 promoter reporter plasmid (-1609pLuc) and various amounts Sp2 expression vector or control vector pcDNA3.1 as indicated. The luciferase activity is presented as a percentage of that of control plasmid transfected cells. The data are presented as the mean ± S. E. of three independent experiments. (B) Increased expression of Sp2 inhibited endogenous CEACAM1 expression in NbE cells. NbE cells were transfected with control expression vector pcDNA3.1 or pcDNA-Sp2 expression vector. Twenty four hours after transfection, the cells were harvested and lysed in RIPA buffer. Equal amounts of proteins were loaded and electrophoresed on an 4-12% SDS-

polyacrylamide gel. Western immunoblot analysis was performed by using anti-Sp2 antibody and anti-CEACAM1 antibody (Ab669). The expression of α-actin was used as control. (C) Mutation of Sp2 binding site increases CEACAM1 promoter activity. Mat-LyLu cells were transfected with luciferase reporter plasmids containing wild-type CEACAM1 promoter (-194pLuc) or CEACAM1 promoter with mutation of Sp2 binding site (-194mutpLuc). Fold induction of luciferase activity was calculated relative to that of –194pLuc. (D) Association of Sp2 with CEACAM1 promoter in vivo. ChIP analysis of the CEACAM1 gene in NbE and Mat-LyLu cells was performed with anti-Sp2 antibody. PCR was used to detect the nt –162 to –240 region of the CEACAM1 promoter. PCR of histone 3.3 DNA was used as a control for total chromatin input.

Fig. 6. (A) Inhibition of HDAC activity by TSA activates CEACAM1 promoter activity. Mat-LyLu cells were transfected with –194pLuc or -194mutpLuc in the presence or absence of 1 uM trichostatin (TSA). Fold induction of luciferase activity was calculated relative to that of –194pLuc in the absence of TSA. (B) PCR of DNA fragments immunoprecipitated by anti-AcH4 antibodies.

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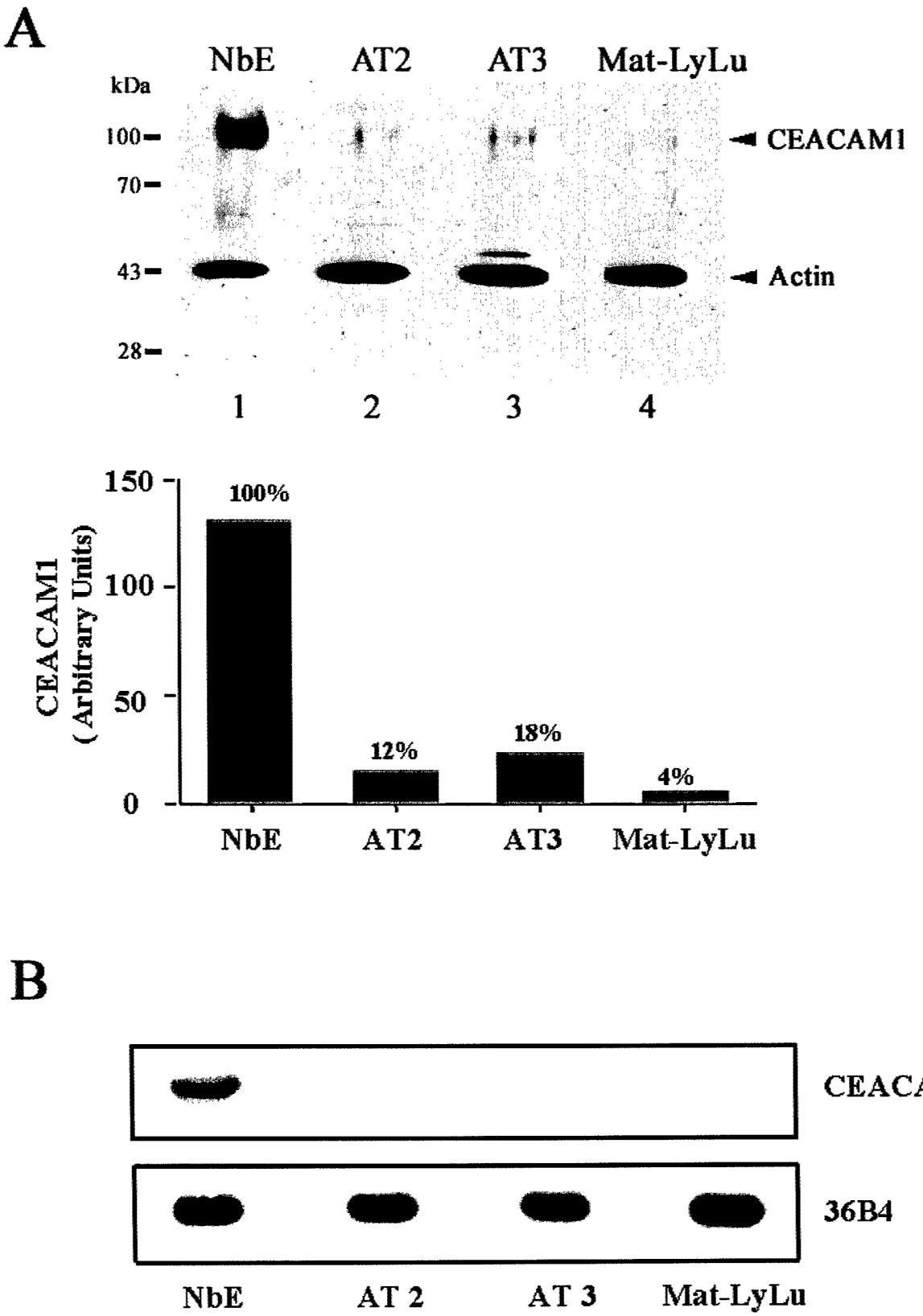
Fig. 1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100%
12%
18%
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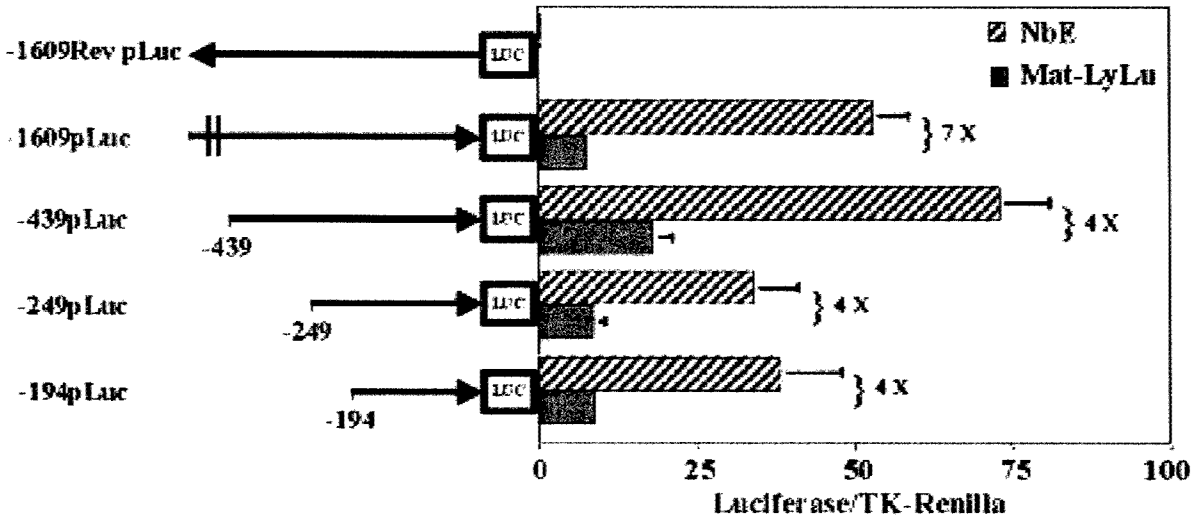
Fig. 2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Luciferase/TK-Renilla

Fig. 3

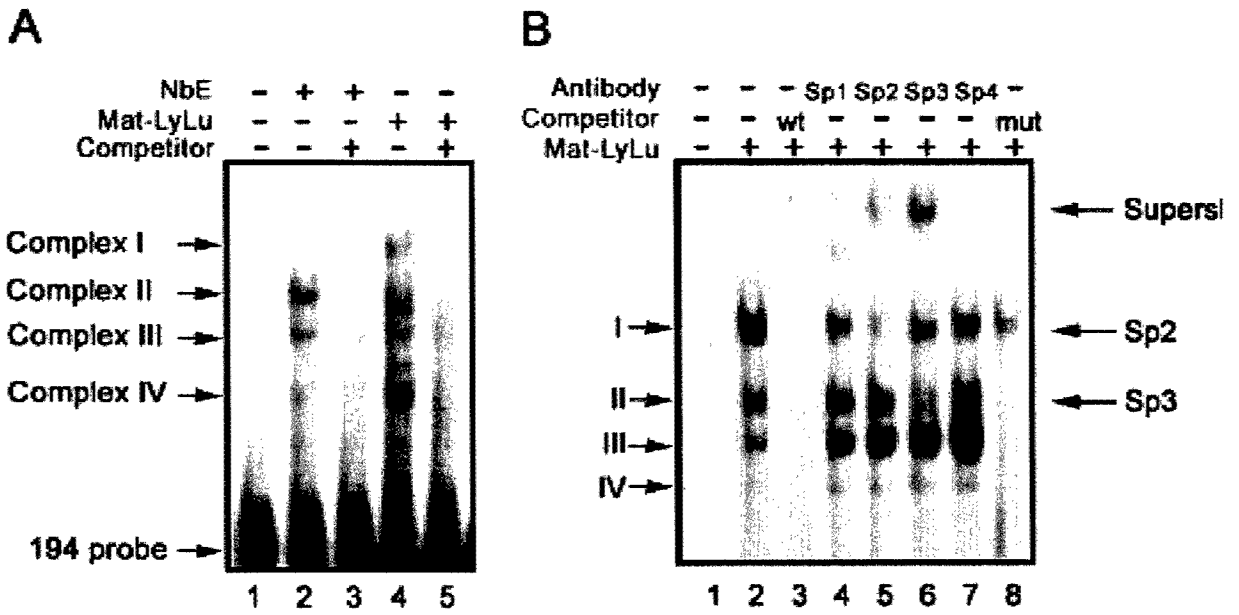
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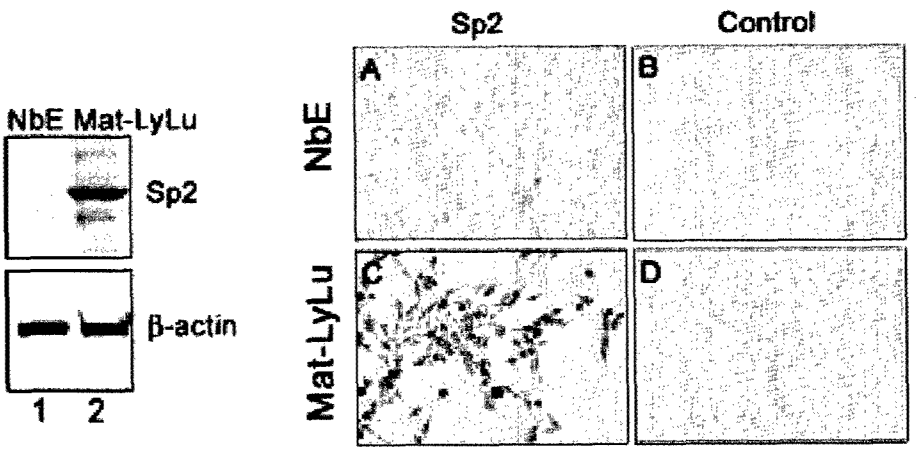
Fig. 4



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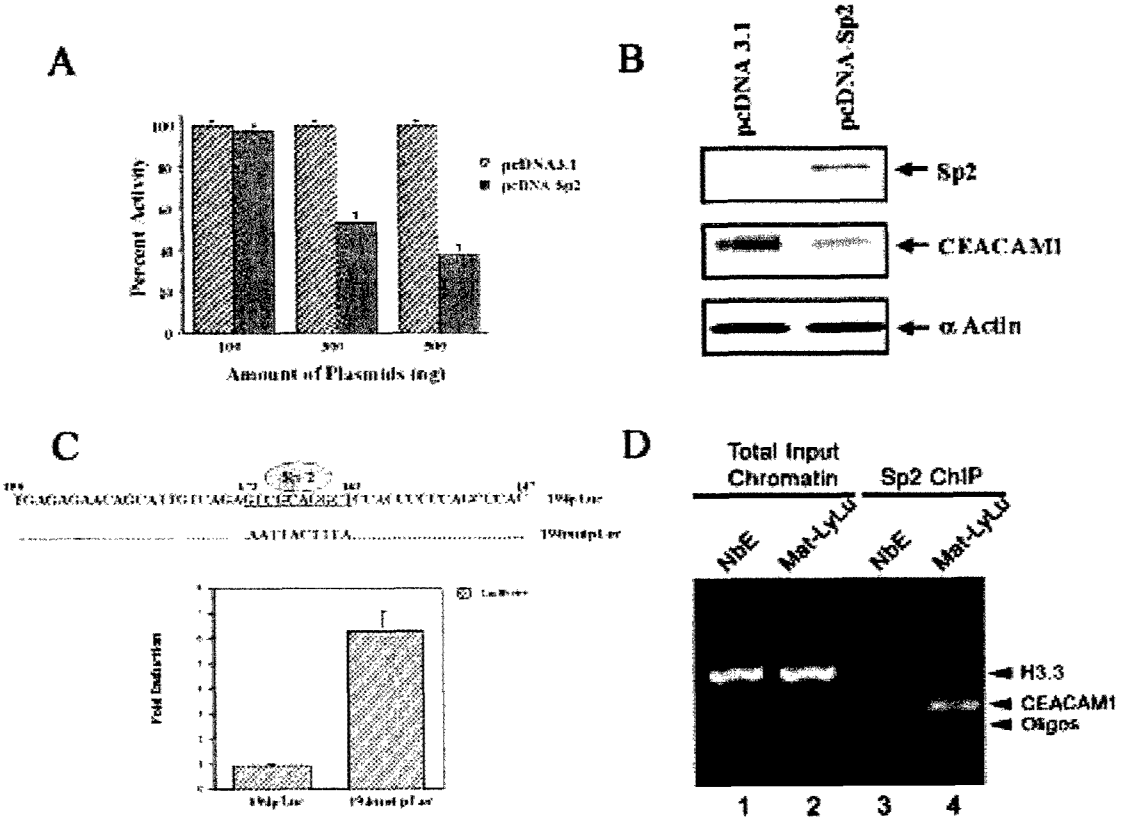
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Amount of Plasmids (ng)
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Sp2
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C
Sp 2
Fold Induction
D
Total Input Chromatin
Sp2 ChIP
NbE
Mat-LyLu
H3.3
CEACAM1
Oligos
1
2
3
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Fig. 6

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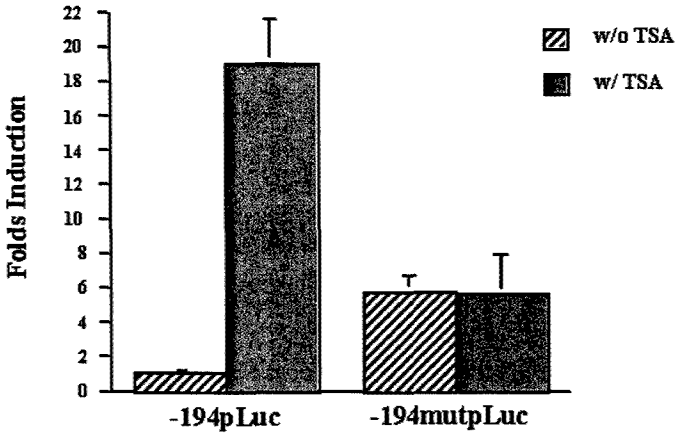
A



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